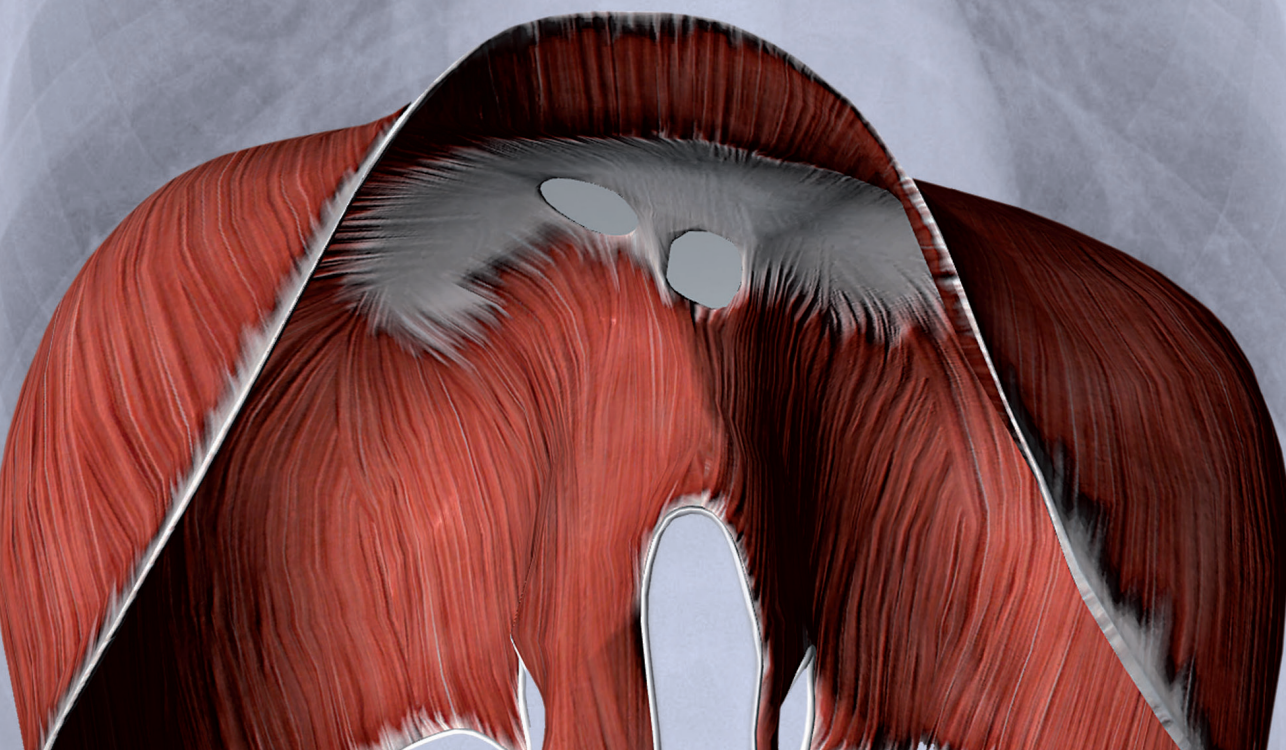
RESPIRATORY MUSCLE WEAKNESS IN CRITICAL ILLNESS

from pathophysiology to therapeutic interventions

Willem-Jan Schellekens



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Contents

Chapter 1

Introduction and outline

1. Respiratory muscle weakness and critical illness

Mechanical ventilation in a patient with acute respiratory failure is a life saving intervention. In most of these patients mechanical ventilation can be discontinued as soon as the underlying reasons for acute respiratory failure have been resolved. However, in 20 – 30 % of ventilated critical ill patients, weaning from mechanical ventilation is difficult [1, 2]. In a substantial proportion of patients on the ventilator respiratory muscle weakness develops, which is associated with difficult weaning [3]. It should be noted that prolonged weaning is associated with adverse clinical outcome, including mortality [3-8]. In addition to prolonged weaning, respiratory muscle weakness is associated with increased hospital length of stay [8]. Respiratory muscle weakness develops rapidly in patients on controlled mechanical ventilation. For instance, Jaber and colleagues demonstrated that pressure generation of the diaphragm is decreased by ±25% after 3-4 days of controlled mechanical ventilation [9]. More recently it was demonstrated that high level of ventilator assist is associated with the development of diaphragm muscle atrophy and dysfunction [10].

Since respiratory weakness is associated with prolonged mechanical ventilation, it has a tremendous financial impact on societies [3, 8, 11, 12]. Off all patients receiving mechanical ventilation, 6% of those patients receive prolonged mechanical ventilation (>7 days), but consume about a third of intensive care unit resources [3, 13]. In the United States in 2008, approximately 377.000 critically ill patients required ventilator support for more then 4 days and this is expected to increase up to 625.000 in 2020 [14]. This indicates that society resources for treatment of these critically ill patients are about to double in a time frame of 12 years in Western society. Since respiratory muscle weakness plays a key role in difficult weaning, it is of vital importance towards patients, health care providers and societies to further unravel pathophysiology of respiratory muscle weakness in critical illness and explore potential therapies.

2. Origin of respiratory muscle weakness in critical illness

The pathophysiology of respiratory muscle weakness in the critically ill is incompletely understood, but proposed to be multifactorial [2, 15, 16]. Theoretically, it may result from modifications at any point between the respiratory centres in the brainstem and muscle contractile proteins. At the muscle level, weakness may result from loss of tissue (atrophy) or dysfunction of the remaining muscle fibers. Indeed, in critically ill patients both atrophy and muscle fiber dysfunction have been reported as summarized in figure 1 and further discussed below.

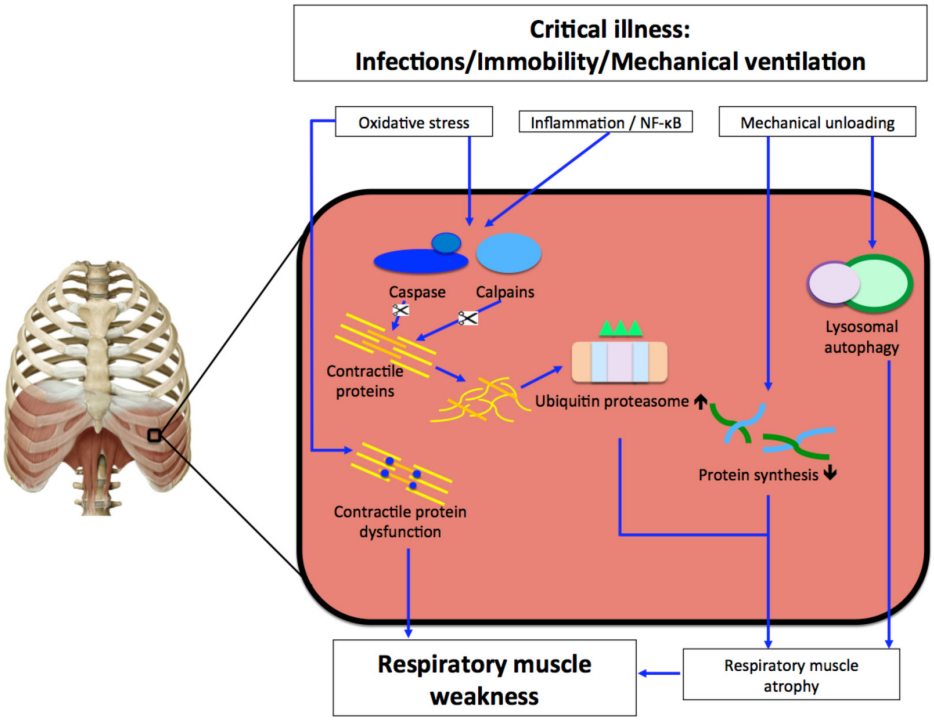

Figure 1 *Proposed mechanisms of respiratory muscle weakness (as published in chapter 7).*

2.1 Respiratory muscle atrophy

To maintain muscle mass, proteolysis and protein synthesis should be balanced [15]. Either an increase in proteolysis or decrease in protein synthesis will result in atrophy. It has been demonstrated that in patients on controlled mechanical ventilation respiratory muscle atrophy develops rapidly [17]. Today, there are data, which demonstrate that indeed both proteolysis is increased and protein synthesis is decreased in the diaphragm of ventilated ICU patients. A central pathway in the development of this respiratory muscle atrophy is the ubiquitin-proteasome pathway (figure 2) [17, 18].

This pathway can be triggered by oxidative stress [19, 20], calpains and caspases [9, 19, 21], decrease in p-AKT [22, 23] and cytokines [24]. The ubiquitin-proteasome is the main system of proteolysis for non-lysosomal break down of cell proteins in eukaryotic cells [25]. In this pathway, released myofibril proteins are labelled by adding ubiquitin chains by E3-ligases MuRF-1 and MAFbx. With these labels proteins are recognized for further degradation by the proteasome complex [25, 26]. Large skeletal muscle proteins like actomyosin have to be cleaved from the

sarcomere before further breakdown is possible in the ubiquitin-proteasome pathway [27]. Caspase-3 [21, 27, 28] and calpains [9, 29] have been suggested to play a prominent role in cleaving muscle proteins from the sarcomere.

Another important regulating pathway in skeletal muscle is the autophagy-lysosome pathway [24]. This autophagy-lysosome pathway is continuously active in cells and can be up regulated under certain conditions, such as oxidative stress or mechanical ventilation [30, 31].

Despite the clues that the autophagy-lysosome pathway and the ubiquitin-pathway are activated in skeletal muscles during critical illness, it is unknown to what extent proteolysis contributes to a reduced force-generating capacity of the diaphragm muscle.

As mentioned earlier, decreased protein synthesis may contribute to the development of atrophy. In ventilated animals, after only six hours of controlled mechanical ventilation contractile protein synthesis is reduced [32]. In healthy volunteers, LPS infusion markedly decreased skeletal muscle protein synthesis [33]. Moreover, in skeletal muscle of critical ill patients contractile protein synthesis was found to be reduced [34, 35], suggesting a role for reduced protein synthesis in the development of respiratory muscle weakness in the critically ill.

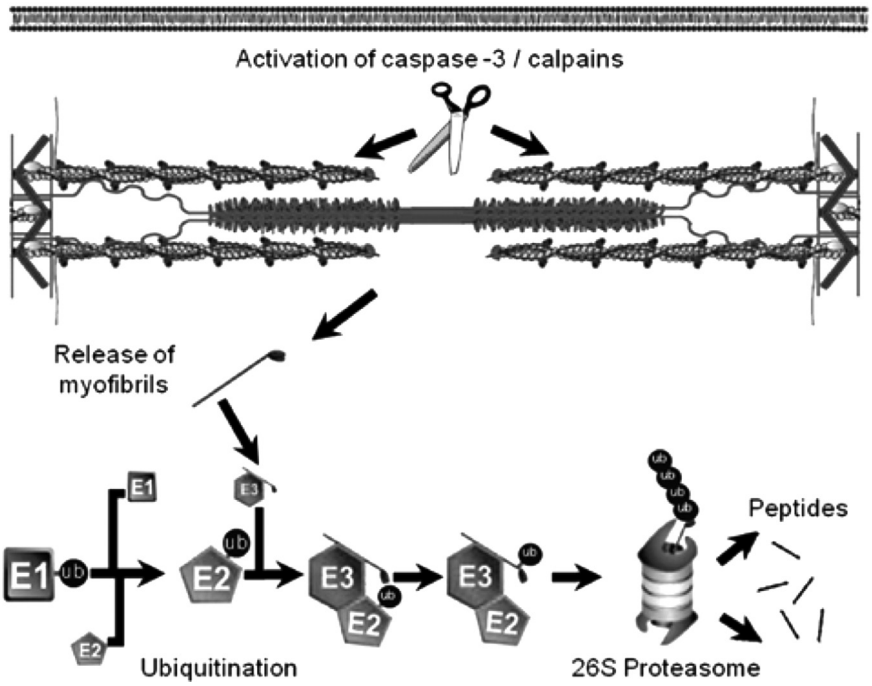

Figure 2 *Schematic view of ubiquitin-proteasome pathway.*
Adapted from Jackman, American Journal of Physiology, Cell Physiology 2004.

2.2 Oxidative and nitrosative stress

Oxidative stress has been shown to modify and damage respiratory muscle proteins [36]. These modifications may result in contractile protein dysfunction [37]. Also, damaged contractile proteins are prone for degradation [26]. Thereby oxidative stress can cause respiratory muscle atrophy during mechanical ventilation [19, 20] and endotoxemia [36]. One of the proteolytic pathways that is activated by oxidative stress, is the ubiquitin-proteasome pathway [26, 38]. In addition, enhanced production of the reactive nitrogen species nitric oxide (NO) and peroxynitrite may result in nitrosative stress [39, 40]. During nitrosative stress, contractile proteins may be nitrosylated, which could affect contractile function [41].

2.3 Inflammatory pathways

Evidence has accumulated that inflammatory pathways are involved in the development of respiratory muscle atrophy in critically ill patients [15]. Pro-inflammatory cytokines, such as IL-6 induce muscle atrophy [42, 43]. The ubiquitous transcription factor Nuclear Factor KappaB (NFκB) plays a key role in activation of the inflammatory response and activation of proteolytic pathways [44, 45]. For example NFκB enhances the expression of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis-α [46, 47]. Moreover, NFκB activates the E3 ligase MuRF-1, which is involved in the proteolytic ubiquitin proteasome pathway [48]. NFκB can be activated by a variety of upstream signals including bacterial products like lipopolysacharide (LPS), pro-inflammatory cytokines and oxidative stress [49]. Exposing respiratory muscles to LPS increases expression of several pro-inflammatory cytokines via NFκB [50]. LPS-induced activation of NFκB is known to be mediated through Toll Like Receptor(TLR)4 [47, 51]. However, it is unknown if inhibiting TLR4 prevents the development of ventilator-induced diaphragm atrophy.

2.4 Contractile protein dysfunction

Besides a reduction in contractile protein content, respiratory muscle weakness can develop as consequence of contractile protein dysfunction. Posttranslational modifications of contractile proteins may occur as a result of oxidation, nitrosylation or phosphorylation [16, 20, 37, 52-54]. Due to these posttranslational modifications, contractile proteins may become less sensitive to changes in intracellular calcium concentration (reduced calcium sensitivity) [16, 54]. However, the effect of mechanical ventilation on contractile protein function has not been evaluated.

3. Aims and outline of the thesis

The overall aims of this thesis are 1. to improve the understanding of ventilator induced respiratory muscle dysfunction and 2. to evaluate possible strategies that may improve respiratory muscle function during mechanical ventilation.

Studies in humans and animal models have demonstrated that diaphragm weakness develops during mechanical ventilation, but the underlying pathophysiology is incompletely understood. It is known however, that respiratory muscle weakness induced by mechanical ventilation results from modifications at the muscle fiber level. For example, decreased cross sectional area was reported after mechanical ventilation in both rodents and ICU patients [17, 32]. Furthermore, in muscle bundles obtained from the diaphragm of ventilated animals decrease in force generation exceeds the loss of cross sectional area (reduced specific force) [55, 56]. However, it is unknown if reduced specific force (force corrected for cross sectional area) following mechanical ventilation can be explained by loss of diaphragm contractile proteins. Notably, next to atrophy, myofibrillar damage has been reported in the ventilated diaphragm [9, 55]. The underlying mechanisms for the development of muscle injury are obscure, but it is known that passive elastic properties are important determinants for sarcomere stability during force generation [57]. The giant muscle protein titin is the major factor for optimal passive elastic properties of skeletal muscles [58]. Titin contains a spring-like molecular structure, the PEVK segment that largely determines its mechanical properties. Posttranslational modifications of this PEVK segment, for instance by phosphorylation, may affect passive mechanical characteristics of the muscle fiber. Today, the effects of mechanical ventilation on passive elastic force generation and titin structure are unknown. In ***chapter 2*** we therefore investigated the consequences of mechanical ventilation on diaphragm structure and function. To this end, diaphragm was analysed for contractile protein content and titin content. Diaphragm function was studied for active force and passive force upon stretch. The effect of titin phosphorylation status on passive force generation was evaluated.

Sepsis is an important risk factor for the development of ICU-acquired weakness [59]. Previous studies in septic rodents have shown that skeletal muscle protein breakdown is increased due to activation of the ubiquitin-proteasome pathway [60]. Circulating ligands may induce skeletal muscle protein breakdown during sepsis [61, 62]. However, it is unknown if ligands in plasma of septic patients can activate the ubiquitin-proteasome pathway and so induce loss of skeletal muscle protein. In ***chapter 3*** the effects of plasma derived from patients with septic shock

was studied on cultured skeletal myotubes. To determine if plasma from septic patients induces loss of muscle protein we examined contractile protein content in these cultured skeletal muscle cells. With that, inflammatory pathways and components of the ubiquitin-proteasome pathway were studied.

Mechanical ventilation can induce a pulmonary inflammatory response, which after decompartmentalization may result in a systemic inflammatory response [63]. TLR receptors, present in the lung, are essential for initiation of an inflammatory response, for instance by recognizing specific ligands like microbial components, but also proteins from injured tissue [64, 65]. TLR4, a subtype of TLR receptors expressed on muscle, can upregulate inflammatory genes in the diaphragm of rodents after exposure to specific ligands [66]. However, it is unknown if mechanical ventilation induces diaphragm TLR4 activation and if so, induces an inflammatory response in the diaphragm. Therefore, we studied in ***chapter 4*** the role of TLR4 signaling in ventilator-induced diaphragm atrophy. In this study wild-type and TLR4 deficient animals were subjected to controlled mechanical ventilation. Diaphragm was thereafter analysed for contractile protein content, pro-inflammatory cytokines and markers of autophagy.

In critically ill patients on the ventilator, hypercapnic acidosis is an accepted side effect of the so-called lung protective mechanical ventilation strategy ("*permissive* hypercapnia"). Recently it was proposed that hypercapnic acidosis itself has beneficial effects ("*therapeutic* hypercapnic acidosis"), such as dampening of the inflammatory response and limiting ventilator-induced lung injury [67-69]. However, it is unknown whether hypercapnic acidosis protects the diaphragm from the deleterious effects of controlled mechanical ventilation. In ***chapter 5*** the effects of acute hypercapnic acidosis on the respiratory muscles was evaluated in mechanically ventilated animals. Diaphragm muscle was analysed for contractility and several biochemical markers for inflammation, proteolysis and oxidation.

Mechanical ventilation and experimental sepsis have been shown to enhance nitrosative and oxidative alterations in diaphragm muscle and are associated with dysfunction [20, 70]. Accordingly, strategies that aim to reduce nitrosative, oxidative and inflammatory stress may protect the diaphragm during critical illness. Levosimendan is a relatively new cardiac inotrope that enhances calcium sensitivity of cardiac muscle. Interestingly, preliminary data suggest that levosimendan exhibits anti-inflammatory and anti-oxidative properties as well [71-74]. For example, levosimendan decreases IL-1β levels during experimental sepsis in rodents [73]. In-vitro, levosimendan reduces LPS-induced upregulation of IL-6, nitrite production and inducible nitric oxide synthase expression [71]. Although

levosimendan has been shown to improve respiratory muscle function [75], the effects of levosimendan on inflammatory and nitrosative markers have not been studied in the diaphragm. Therefore, in ***chapter 6*** the effects of levosimendan on the diaphragm in a model of systemic inflammation (endotoxemia) under mechanical ventilation was studied.

Finally, ***chapter 7*** reviews the current knowledge regarding potential strategies to optimize respiratory muscle function in the critically ill patient.

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Chapter 2

Titin and diaphragm dysfunction in mechanically ventilated rats

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ABSTRACT

Purpose Diaphragm weakness induced by mechanical ventilation may contribute to difficult weaning from the ventilator. For optimal force generation the muscle proteins myosin and titin are indispensible. The present study investigated if myosin and titin loss or dysfunction are involved in mechanical ventilation induced diaphragm weakness.

Methods Male Wistar rats were either assigned to a control group (n=10) or submitted to 18 hours of mechanical ventilation (MV, n=10). At the end of the experiment, diaphragm and soleus muscle were excised for functional and biochemical analysis.

Results Maximal specific active force generation of muscle fibers isolated from the diaphragm of MV rats was lower than controls (128±9 versus 165±13 mN/mm^2, p=0.02) and was accompanied by a proportional reduction of myosin heavy chain concentration in these fibers. Passive force generation upon stretch was significantly reduced in diaphragm fibers from MV rats by ~35%. Yet, titin content was not significantly different between control and MV diaphragm. In vitro pre-incubation with phosphatase-1 decreased passive force generation upon stretch in diaphragm fibers from control, but not from MV rats. Mechanical ventilation did not affect active or passive force generation in the soleus muscle.

Conclusions Mechanical ventilation leads to impaired diaphragm fiber active force generating capacity and passive force generation upon stretch. Loss of myosin contributes to reduced active force generation, while reduced passive force generation is likely to result from a decreased phosphorylation status of titin. In the soleus muscle of 18 hours mechanically ventilated rats these impairments were not discernable.

INTRODUCTION

Mechanical ventilation is a life-saving intervention in critically ill patients, but comes with several adverse events including weakness of the diaphragm muscle [1, 2]. This is clinically important as weakness of the inspiratory muscles plays an important role in difficult weaning from mechanical ventilation [3-5]. Several studies have indicated that respiratory muscle weakness induced by mechanical ventilation primarily results from changes within the diaphragm muscle fibers. For example, diaphragm fibers isolated from patients and rodents that underwent controlled mechanical ventilation show decreased cross-sectional areas, i.e. atrophy [6-8], which most likely affects force generating capacity of the diaphragm. Moreover, studies on isolated diaphragm bundles from mechanically ventilated animals demonstrate reduced generation of *specific* force, i.e. force per cross-sectional area [9-12]. This either suggests that contractile protein concentration is reduced or that remaining contractile proteins have reduced functionality. Measuring contractile function of permeabilized muscle single fibers is the most appropriate technique to study the involvement of contractile protein dysfunction [13]. Force generating capacity of permeabilized muscle fibers strongly depends on the content of the contractile protein myosin [14]. Accordingly, the first aim of the present study was to investigate whether reduced force generating capacity of diaphragm muscle fibers from mechanically ventilated rats is associated with decreased myosin concentration.

In addition to atrophy, signs of muscle fiber injury like myofibrillar disarray and Z-band streaming have been observed in the diaphragm of mechanically ventilated humans and animals [8, 9, 15, 16]. The mechanisms leading to muscle fiber injury are largely unknown. For structural stability and optimal active force generation, passive elastic structures are indispensible [17]. Titin is the major determinant of passive-elastic properties of striated muscle [18]. In an animal model of peripheral muscle disuse a preferential loss of titin explained abnormal sarcomeric organization [19]. Passive elastic properties of skeletal muscle fibers can also be modulated by posttranslational modifications or alternative splicing of titin [20], as occurs in diaphragm fibers from patients with COPD [21]. The effect of mechanical ventilation on titin function in respiratory muscles is unknown. Accordingly, the second aim of the present study was to investigate the passive elastic properties of permeabilized diaphragm fibers from mechanically ventilated rats.

Previous studies indicated that the diaphragm is more susceptible to the deleterious effects of mechanical ventilation than peripheral skeletal muscles [6, 7, 10]. Whether the effects of mechanical ventilation on muscle protein function are different between respiratory and peripheral muscle is currently unknown. Therefore, we additionally determined active and passive force generation of soleus muscle fibers from the same animals. We hypothesized that mechanical ventilation induces loss of myosin and titin, resulting in reduced active and passive force generation of diaphragm muscle fibers. We expected the impairments to be less prominent in soleus muscle fibers.

MATERIALS & METHODS

Experimental design

Two groups of male Wistar rats (body weight ~300 g) were studied, controls (n=10) and after 18 hours of mechanical ventilation (MV, n=10). Five to six isolated skinned single muscle fibers from the diaphragm and the soleus of each animal were investigated on cross-sectional area, myosin heavy chain isoform and concentration, calcium-induced maximal *active* force generation and *passive* force generation upon stretch (total number of approximately 60 fibers per group). Titin content was studied in diaphragm homogenates and in diaphragm cryosections. Since impaired passive force generation upon stretch in diaphragm fibers of MV rats was not accompanied by reduced titin content, we subsequently examined the effect of post-translational dephosphorylation on passive force generation of the rat diaphragm. All experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care.

Animal model

Our animal model of mechanical ventilation was based on previously described methods [11], with minor modifications.

Skinned fiber contractile measurements

Maximal active force generation and passive force generation upon stretch of skinned single fibers isolated from the diaphragm muscle were determined as described previously [22].

Effect of post-translational dephosphorylation

The effects of post-translational dephosphorylation on passive force generation were investigated according to previous described methods [23]. Skinned

diaphragm bundle preparations were incubated with phosphatase-1 (PP-1; 1.5U/µl) in relax solution for two hours at room temperature. Subsequently single fibers were isolated and passive tension upon stretch was measured.

Myosin heavy chain isoform and concentration
Determination of myosin heavy chain isoform composition and content by SDS-PAGE was described previously [24] and adapted from Geiger et al. [14]. Since only 5 diaphragm fibers from each group expressed the slow isoform of myosin heavy chain, these were excluded from further analysis. Accordingly all fibers were classified as type II.

Titin content
Content of the major sarcomeric protein titin was determined by SDS-agarose gel electrophoresis and by immunohistochemistry as described previously [21].

Statistical methods
Sample size of approximately 50 fibers per group, calculated a priori, was based upon the assumption to detect a reduction of passive force of at least 25%, with a standard deviation of 50% [21], 80% probability and an alpha level of 0.05. Differences between MV and control rats regarding maximal force generation, myosin heavy chain concentration and titin content were analyzed with Student t-tests. Repeated-measures analysis was performed with post hoc Student t-testing at each fiber length to evaluate the statistical significance of differences in single fiber passive-tension data between groups. A probability level of $p<0.05$ was considered significant. Mean ± SE values are presented in text, tables and figures.

RESULTS

Animal characteristics
MV rats exhibited stable hemodynamics during 18 hours mechanical ventilation (mean arterial pressure; 99±1 mmHg) and pH, PaO_2, and $PaCO_2$ were normal at the end of the experiment (7.48 ± 0.02, 13.4 ± 0.8kPa and 5.5 ± 0.2 kPa respectively).

Active force generation
The cross-sectional area of skinned diaphragm fibers from MV animals was ~25% lower than in control animals (2.0±0.1 versus 2.6±0.2 $x10^{-3}$ mm^2, p=0.002), which is consistent with the development of muscle fiber atrophy. Moreover, mechanical ventilation significantly reduced diaphragm fiber force generating capacity, even

when corrected for loss of cross-sectional area (p=0.02, Figure 1).
In soleus muscle fibers mechanical ventilation did neither affect cross-sectional area (2.4±0.1 versus 2.6±0.2 $x10^{-3}$ mm^2, for MV and control rats) nor specific force generating capacity (136±6 versus 127±12 mN/mm^2 for control and MV rats).

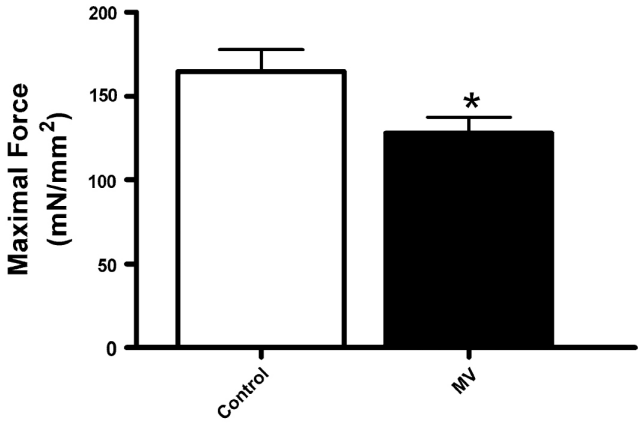

Figure 1 Maximal active specific force generation of diaphragm fibers from control and mechanically ventilated rats (MV). * p<0.05.

Myosin heavy chain concentration and active force
Myosin heavy chain concentration was ~25% lower in diaphragm fibers from MV animals than in diaphragm fibers from control animals (p=0.03, Figure 2). Maximal force generation per myosin molecule, i.e. maximal force divided by myosin heavy chain content, was not significantly different between MV diaphragm fibers and control (2.31±0.26 and 2.97±0.55 N/mg respectively, p=0.3)
Myosin heavy chain concentration in soleus muscle fibers was not significantly affected by mechanical ventilation (80±12 versus 79±10 µg/µl for control and MV animals respectively).

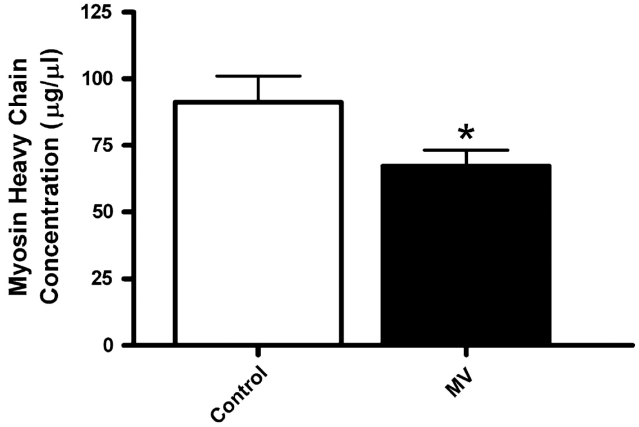

Figure 2 Myosin heavy chain concentration in diaphragm fibers from control and mechanically ventilated rats (MV). * p<0.05.

Passive force generation

Passive force generation upon stretch was impaired in diaphragm fibers from MV animals (Figure 3). At fiber lengths longer than 120% of optimal length, diaphragm fibers from MV animals displayed significantly lower passive forces compared to fibers from control rats.

In soleus muscle fibers passive force generation was not affected by mechanical ventilation. For example, at 140% of optimal fiber length soleus fibers generated passive forces of 47±3 versus 51±4 mN/mm^2 for control and MV animals respectively.

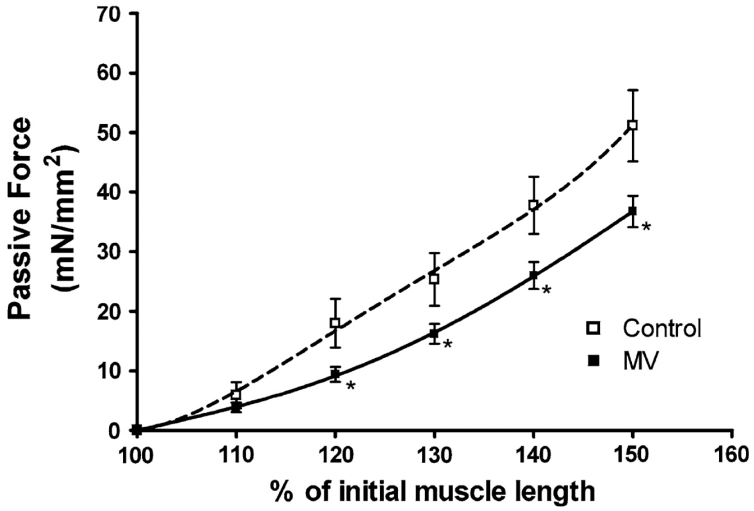

Figure 3 Passive force generation at different muscle fiber lengths of diaphragm fibers from control and mechanically ventilated rats (MV). Lines represent fourthorder polynomial fits. *p<0.05.

Titin content

Reduced passive tension of skeletal muscle fibers can result from loss of titin, increased expression of longer titin molecules or posttranslational modifications of titin [20].

Titin content analyzed by SDS-agarose gel electrophoresis was not significantly different in diaphragm samples from MV animals compared to control (Figure 4). Furthermore, the content of titin's degradation product T2 in the diaphragm was not significantly different between MV and control rats (2.7±0.5 versus 4.0±0.9 AU/µg muscle weight, p=0.23), which excludes titin breakdown as a reason for lower passive force in MV fibers. To gain insight into the size of titin in diaphragm muscle of MV rats, we studied the mobility of titin on gel. A representative gel shows that the mobility of titin from rat diaphragm samples is distinctly faster than the mobility of titin from human soleus muscle, which is explained by a

smaller molecule size. The mobility of titin was not different between diaphragm samples from MV rats and controls, indicating the absence of detectable size differences in titin.

Immunohistochemical analysis confirmed the absence of titin loss in the diaphragm of MV rats. Staining intensities of antibodies directed against the titin epitopes T12 (Z-line) and T51 (M-line) were comparable between MV and control diaphragm (Figure 5).

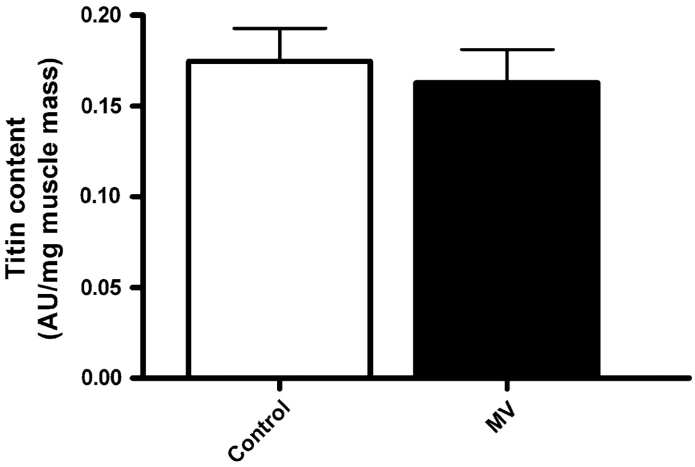

Figure 4 Titin content in diaphragm samples from control and mechanically ventilated rats (MV).

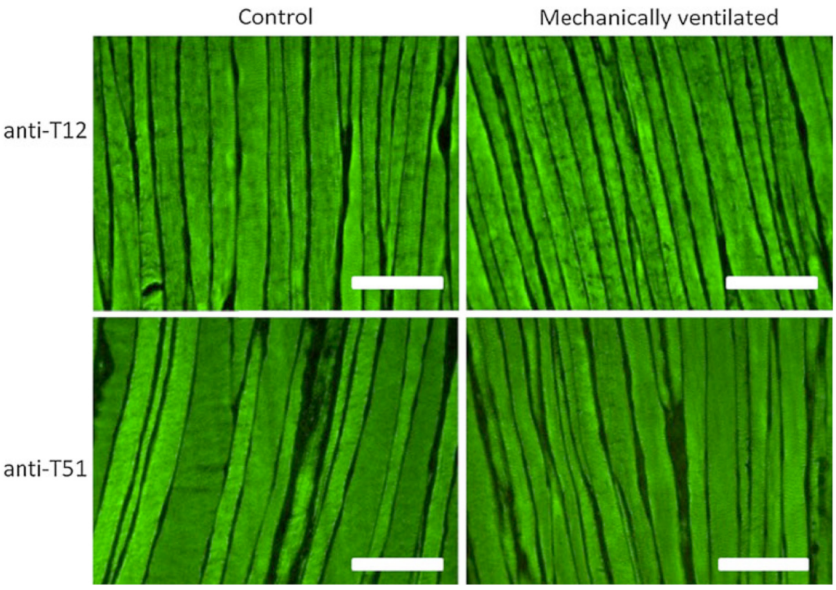

Figure 5 Representative photographs of cryosections of the diaphragm from control and mechanically ventilated rats stained with antibodies against two specific titin epitopes T12 (Z-line) and T51 (M-line). Bar = 100 µm.

Passive force and dephosphorylation

As titin content and size were not affected by mechanical ventilation, we investigated if posttranslational modification of titin could explain increased compliance of diaphragm fibers in MV animals. Recent studies have shown that titin's compliance can be increased by lowering the phosphorylation state of titin [23]. Accordingly, we examined the effect of dephosphorylation on passive force generation of diaphragm fibers from control and MV rats. Figure 6 shows that pre-incubation with PP-1, a dephosphorylating enzyme, significantly reduced passive force generation upon stretch in diaphragm fibers from control animals. However, PP-1 did not affect passive force generation in diaphragm fibers of MV rats.

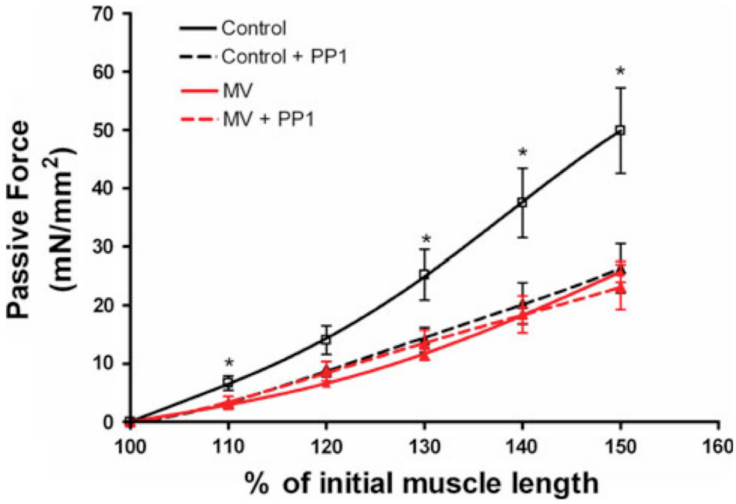

Figure 6 Effect of dephosphorylation on passive force generation at different muscle fiber lengths of diaphragm fibers from control and mechanically ventilated rats (n=5 fibers per group). Lines represent fourth-order polynomial fits. Dotted lines: fibers were pre-incubated with phosphatase-1 (PP1, 1.5U/µl, for two hours). * $p<0.05$ vs control + PP1.

DISCUSSION

Previous studies have reported that controlled mechanical ventilation has profound effects on structure and force generating capacity of the inspiratory muscle in humans and animal models [1, 6-8, 10, 12]. The data of the present study provide the following new and important insights in the effects of 18 hours controlled mechanical ventilation on diaphragm muscle function; (1) in addition to atrophy, decreased myosin concentration contributes to reduced active force

generation, (2) mechanical ventilation significantly reduces passive force of diaphragm muscle fibers, (3) the effects of mechanical ventilation on passive force can be mimicked by dephosphorylating the elastic protein titin and (4) the effects of mechanical ventilation on active and passive force do not occur in the soleus muscle within this time frame.

Reduced force generation and loss of myosin

Several studies, both in humans and in animal models, showed that mechanical ventilation rapidly induces atrophy of diaphragm muscle fibers [1, 6-8]. Our results are in line with those studies as we found that 18 hours mechanical ventilation provoked a 20% reduction of diaphragm fiber cross-sectional area. The capacity of a muscle to generate force strongly depends on the number of cross-bridges that can be formed in parallel [14]. As such, reduction of diaphragm muscle fiber cross-sectional area may explain reduced diaphragm force output when cross-sectional area and contractile protein content decrease proportionally. Yet, reduction of cross-sectional area only partially explains mechanical ventilation-induced diaphragm weakness, since several studies have shown that prolonged mechanical ventilation reduces diaphragm bundle or single fiber *specific* force generating capacity, i.e. absolute force divided by cross-sectional area [9-12, 25]. Our data are in line with these studies and show that fiber *specific* force generating capacity is already reduced after 18 hours of mechanical ventilation (figure 1). Moreover, we found that myosin concentration is reduced in diaphragm fibers from mechanically ventilated animals (figure 2), indicating that the severity of myosin loss exceeds the degree of fiber atrophy. This can explain the large decrease in contractile protein content recently observed in the diaphragm of mechanically ventilated humans [26]. Notably, after correction of force for myosin concentration, no differences exist in force between control and ventilated diaphragm. This implies that contractile protein loss is a strong determinant of diaphragm weakness upon mechanical ventilation. In addition, this finding indicates that the remaining contractile proteins display normal maximal force generating capacity. This is in contrast to the diaphragm in patients with COPD, where loss of force results from reduction in protein content and impaired function of the remaining contractile protein [27, 28]. Contractile protein loss can result from both increased proteolysis as well as reduced synthesis. Indeed, previous studies have demonstrated that synthesis of contractile proteins is reduced [29], and proteolytic systems like the ubiquitin-proteasome pathway, caspase-3, calpains and lysosomes are activated in the diaphragm of mechanically ventilated rodents and humans [6, 7, 12, 26, 30, 31].

Titin function modulation due to mechanical ventilation: causes and consequences

Passive elastic structures within muscle are indispensible for structural stability and optimal active force generation [17]. Titin is the most important determinant of passive-elastic properties of striated muscle fibers [18]. The current study demonstrates that mechanical ventilation reduces stiffness of diaphragm muscle fibers. As disuse may enhance titin degradation [19], we anticipated that reduced stiffness of diaphragm fibers from mechanically ventilated animals resulted from the loss of titin. Surprisingly, both agarose gel electrophoresis and histochemical analysis demonstrated that titin content in diaphragm fibers is not affected by mechanical ventilation. Alternatively, reduced elasticity of muscle fibers can result from post-translational modifications of titin [32]. The part of titin that largely determines its elastic properties is the PEVK (Pro-Glu-Val-Lys) segment, which encodes a coil-like structure that acts as a molecular spring [33]. Accordingly, post-translational modifications of aminoacids within the PEVK segment may modulate stiffness of the titin molecule. The present findings indicate that titin's stiffness depends on its phosphorylation state, because incubation with the dephosphorylating enzyme phosphatase-1 (PP-1) reduces the stiffness of diaphragm fibers from control animals. This is in line with previous findings in cardiac muscle, which showed that phosphorylation of two constitutively expressed sites within the PEVK segment increased stiffness of isolated cardiomyoctyes [23]. In contrast to control diaphragm fibers, PP-1 did not affect elastic properties of diaphragm fibers from mechanically ventilated rats. These data suggests that the phosphorylation state of titin's PEVK domain in diaphragm fibers from mechanically ventilated animals is already low. Therefore, we conclude that mechanical ventilation decreases the phosphorylation status of titin in diaphragm fibers, resulting in reduced stiffness. Phosphorylation of specific sites in the PEVK domain is controlled by protein kinase C (PKC) [23]. Interestingly, a recent study showed that 3 days of disuse leads to a rapid reduction of PKC activity in peripheral skeletal muscles [34]. Since the diaphragm is contractile inactive during mechanical ventilation, it may be hypothesized that mechanical ventilation reduces PKC activity in the diaphragm.

Alterations in diaphragm muscle stiffness as reported in our study may be linked to muscle protein degradation and synthesis. Proteolytic systems can be activated by the abundance of damaged proteins and indeed, evaluation by electron microscopy revealed sarcomeric injuries in diaphragm fibers of mechanically ventilated humans and animals [8, 9, 15, 16]. Elastic structures inside and outside striated muscle fibers provide sarcomeric stability and minimize the occurrence of sarcomeric injuries. As titin is the main elastic protein within the sarcomere, deletion of titin is known to result in a loss of muscle fiber elasticity and sarcomeric injuries [17]. The loss of stiffness in the diaphragm fibers of ventilated rats may

contribute to the occurrence of saromeric injuries and promote the degradation of contractile proteins, such as myosin. A second consequence of reduced titin stiffness is that it may affect muscle protein synthesis. Evidence exists that titin can act as a mechanosensor regulating protein expression in a sarcomere strain-dependent fashion. Two studies have demonstrate that reduced strain on a kinase domain of titin results in downstream inhibition of muscle protein synthesis [35, 36]. It has therefore been hypothesized that reduced stiffness of titin in the diaphragm fibers from mechanically ventilated animals leads to a lower strain on titin kinase and in this way impedes muscle protein synthesis [37]. This would make sense as reduced strain may occur when the load on the diaphragm is reduced and less muscle mass is required.

Respiratory versus peripheral muscle

Although the respiratory muscles as well as the peripheral muscles are inactive during controlled mechanical ventilation, several studies have demonstrated that the diaphragm is more sensitive to the detrimental effects of mechanical ventilation than peripheral muscles. Diaphragm atrophy in rodents occurs as early as 12 hours of mechanical ventilation [31], while it takes much longer periods of mechanical ventilation to induce significant reductions of peripheral muscle mass [7, 10]. In humans that underwent mechanical ventilation of 18-69 hours, diaphragm fiber cross-sectional area reduced by more than 50%, while pectoralis major fibers did not show any signs of atrophy [6]. Our data is in line with those findings as we did not observe a significant reduction of soleus fiber cross-sectional area in mechanically ventilated animals. More important, our data show that active force and passive force generating capacity of soleus muscle fibers were not affected by mechanical ventilation. So, in contrast to the diaphragm, muscle protein function in the soleus is preserved at least up to 18 hours of mechanical ventilation. We recognize that the comparison of fast-typed diaphragm fibers to slow-typed soleus fibers may be confound by fiber-type specific effects of mechanical ventilation. However, this seems unlikely, because a previous study showed that all diaphragm fiber types developed atrophy upon 18 hours of mechanical ventilation [7].

Clinical relevance

Mechanical ventilation-induced diaphragm dysfunction complicates weaning from the ventilator [3, 4]. Weaning failure is a major clinical problem, as it occurs in a large group of patients undergoing mechanical ventilation. It increases the risk of secondary complications, it prolongs rehabilitation and it puts a huge financial burden on the healthcare system [38]. Although supported modes of ventilation may reduce the development of respiratory muscle atrophy [39],

controlled mechanical ventilation is inevitable in certain patients. In fact, a recent study advocated the use of controlled mechanical ventilation in early ARDS [40]. Unfortunately, no adequate therapies are currently available that improve diaphragm function in these patients. The current findings indicate that loss of myosin and titin dephosphorylation contribute to mechanical ventilation-induced diaphragm dysfunction. The development of treatment strategies aimed at preventing or reversing myosin loss and titin dephosphorylation might therefore be an interesting focus of future studies on mechanical ventilation-induced diaphragm weakness.

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Chapter 3

Plasma from septic shock patients induces loss of muscle protein

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ABSTRACT

Introduction ICU-acquired muscle weakness commonly occurs in patients with septic shock and is associated with poor outcome. Although atrophy is known to be involved, it is unclear whether ligands in plasma from these patients are responsible for initiating degradation of muscle proteins. The aim of the present study was to investigate if plasma from septic shock patients induces skeletal muscle atrophy and to examine the time course of plasma-induced muscle atrophy during ICU stay.

Methods Plasma was derived from septic shock patients within 24h after hospital admission (n=21) and healthy controls (n=12). From 9 patients with septic shock plasma was additionally derived at 2, 5 and 7 days after ICU admission. These plasma samples were added to skeletal myotubes, cultured from murine myoblasts. After incubation for 24h, myotubes were harvested and analyzed on myosin content, mRNA expression of E3-ligase and NFkappaB activity. Plasma samples were analyzed on cytokine concentrations.

Results Myosin content was ~25% lower in myotubes exposed to plasma from septic shock patients than in myotubes exposed to plasma from controls ($p<0.01$). Furthermore, patient plasma increased expression of E3-ligases MuRF-1 and MAFbx ($p<0.01$), enhanced Nuclear Factor kappaB activity ($p<0.05$) and elevated levels of ubiquitinated myosin in myotubes. Myosin loss was significantly associated with elevated plasma levels of IL-6 in septic shock patients ($p<0.001$). Addition of anti-IL-6 to septic shock plasma diminished the loss of myosin in exposed myotubes by ~25% ($p<0.05$). Patient plasma obtained later during ICU stay did not significantly reduce myosin content compared to controls.

Conclusion Plasma from patients with septic shock induces loss of myosin and activates key regulators of proteolysis in skeletal myotubes. IL-6 is an important player in sepsis-induced muscle atrophy in this model. The potential to induce atrophy is strongest in plasma obtained during the early phase of human sepsis.

INTRODUCTION

Skeletal muscle weakness frequently develops in patients admitted to the intensive care unit (ICU), with a reported incidence between 25% and 60% after more than 1 week of mechanical ventilation [1]. ICU-acquired weakness of the respiratory muscles is associated with prolonged weaning. Weakness of peripheral muscles is associated with prolonged rehabilitation [2]. Mortality in patients with ICU-acquired weakness is higher than in ICU patients without weakness [3]. In addition, in survivors the consequences of ICU-acquired muscle weakness may persist for more than one year after ICU discharge [2]. Besides the devastating physical and psychological effects to patients and their family, ICU-acquired weakness has a major economical impact.

Among other factors, sepsis is known to be an important predictor for the development of ICU-acquired muscle weakness [4]. In septic patients, weakness may originate from any point between the central nervous system and the contractile proteins (for review [5]). The effect of sepsis on contractile proteins has received much attention [6,7]. For instance, Tiao et al [6] found that in septic rats, skeletal muscle protein breakdown was increased due to enhanced activity of the proteolytic ubiquitin-proteasome pathway. However, it is unknown whether ligands in human septic plasma can activate the ubiquitin-proteasome pathway and induce loss of myosin. This is of relevance, as it will help to understand the importance of systemic components compared to intrinsic muscle factors, such as disuse, in sepsis-induced muscle atrophy. Previous studies indeed have indicated that cytokines and bacterial cell wall components induce muscle proteolysis in sepsis [8,9], but specific pathways have not been evaluated. Therefore, the objective of the current study was to investigate whether plasma from patients with septic shock induces loss of myosin associated with activation of the ubiquitin-proteasome pathway. We exposed non-diseased cultured skeletal muscle to plasma from patients with septic shock. It was hypothesized that septic plasma induces loss of muscle proteins in highly differentiated skeletal muscle cells. In a follow-up study we investigated the time course of the atrophic response induced by plasma obtained from patients admitted with septic shock. In addition, we explored the relation between myosin loss and plasma levels of inflammatory cytokines.

MATERIALS & METHODS

Experimental design

Two separate sets of experiments were performed. The first set of experiments was conducted to establish whether plasma in the early phase of septic shock induces muscle proteolysis and atrophy. To this end, cultured skeletal muscle myotubes were incubated with either plasma from patients with septic shock obtained within 24 hours of ICU admission or plasma from healthy subjects (controls). After 24 hours of exposure to plasma, skeletal myotubes were harvested for biochemical analysis, including myosin heavy chain content and expression of E3-ligases. The activity of Nuclear Factor kappaB (NFkB) was measured after 1 hour of exposure to plasma.

The second set of experiments was conducted to investigate if the ability of plasma from septic shock patients to induce muscle atrophy changes during the course of ICU admission and if a correlation exists between inflammatory cytokines and loss of myosin. Hence, plasma was derived at different time points during ICU stay of patients with a septic shock and the ability of each plasma sample to induce proteolysis and atrophy in differentiated muscle cells was examined. In addition, plasma levels of cytokines were determined at each time point.

Study population

Twenty-one patients with established septic shock, according to the 2001 International Sepsis Definitions Conference [10], were included in the first set of experiments. Blood was withdrawn from the indwelling arterial catheter within 24 hours of ICU admission. In twelve control subjects blood was obtained through venapuncture.

For the second set of experiments, additional blood samples were obtained from 9 of the 21 septic shock patients at day 2, 5 and 7 of ICU stay. The institutional review board, the medical ethical committee, approved the current study and waived the need for informed consent.

Muscle culture

Muscle cells, C2C12 myoblasts, were cultured into myotubes according to previously described methods [11]. Pilot studies showed stable myosin content in myotubes that differentiated for 7 days and that plasma from healthy subjects does not affect myosin content in these myotubes.

Analysis of myosin content

Myosin content in myotubes was determined by Western blotting according to previous described methods [16].

Analysis of NFkB activity

NFkB is a ubiquitous transcription factor for a variety of cytokines. The DNA binding activity of NFkB has been shown to be highest within several hours after exposure to different stimuli [12,13], which was confirmed by pilot-experiments in our lab. Therefore, in the current study NFkB activity was measured 1 hour after incubation of the myotubes with plasma.

NFkB DNA binding activity was determined by electrophoretic mobility shift assay (EMSA) conform previously described methods [14].

Analysis of E3-ligase mRNA and ubiquitinated myosin levels

Expression of muscle specific E3-ligases, MuRF1 and MAFbx and ubiquitination of myosin was analyzed, conform previously described methods [15].

Cytokines

Plasma levels of IL-6, IFN-γ, TNF-α and IL-1β were measured by enzyme-linked immunosorbent assay (ELISA) (for IL-6, IFN-γ and TNF-α ELISA kits from Sanquin Reagents, Amsterdam, the Netherlands, for IL-1β Quantikinekit from R&D systems, Minneapolis, USA). To further examine the role of IL-6, we first studied myosin content in myotubes that were exposed to septic shock plasma containing an antibody directed against human IL-6 (R&D systems, Minneapolis, USA). In addition, we studied myosin content in myotubes exposed to control plasma containing different concentrations of recombinant human IL-6 (Invitrogen, Carlsbad, California, USA).

Data analysis

Data are presented as means ± standard deviation. The statistical significances of differences regarding myosin content, MuRF-1 and MAFbx expression between myotubes exposed to plasma from controls and patient plasma were analyzed by performing Student t-tests. Differences regarding NFkB activity and cytokine concentrations were statistically tested by performing Mann Whitney U test. Sample size calculations for the follow-up study showed that 9 patients should be sufficient to detect a 25% reduction of myosin content with a standard deviation of 20%, 80% probability and an alpha level of 0.05. One-way ANOVA with post-hoc Bonferroni's multiple comparison testing was used to evaluate whether myosin content, MuRF-1 and MAFbx expression and cytokine levels at each day were statistically different from control. Repeated measures one-way ANOVA with post-hoc Bonferroni's multiple comparison testing was performed to analyze differences between day 0 and subsequent days. The relation between myosin and IL-6 levels was explored using a linear model estimated by Generalized Least Squares to allow for correlations between the measurements caused by repeatedly measuring the same subjects; an unstructured correlation matrix was

used to model this dependence. Since IL-6 levels were not normally distributed, log transformation on these values was applied. P values below 0.05 were considered significant.

RESULTS

Patient characteristics

Patient characteristics are shown in table 1. All patients met septic shock criteria. Thirteen of the 21 patients received 1 bolus of 100 mg hydrocortisone before blood withdrawal. Additional characteristics of patients included in the second set of experiments. None of the control subjects reported any significant past medical history or current use of prescribed medication.

	Control (n = 12)	Septic shock (n=21)
Age (yrs)	59 ± 3	64 ± 3
Male (%)	75% (9/12)	81% (17/21)
APACHE score	NA	21 ± 2
Source of septic shock (%)		
Pulmonary	NA	14% (3/21)
Gastrointestinal		38% (8/21)
Pancreatitis		10% (2/21)
Urinary tract		5% (1/21)
Miscellaneous		33% (7/21)
Past medical history (%)		
COPD	NA	19% (4/21)
Carcinoma		38% (8/21)
Auto-immune disease		19% (4/21)
Diabetes		19% (4/21)

Table 1 Subject characteristics

Effects of early septic shock

Myosin content

Myosin content in skeletal myotubes that were exposed to plasma from septic shock patients was ~25% lower than in myotubes that were exposed to plasma from controls ($p < 0.01$, Figure 1). In addition, the representative Western blot shows that tubulin content was not affected by septic plasma, excluding the involvement of a general protein loss. Furthermore, myosin could not be detected in cell medium (experimental plasma), also excluding cell lysis. Discrimination between patients that did and did not receive steroids prior to blood withdrawal shows that plasma samples from both groups induce a significant and similar myosin loss in myotubes compared to controls.

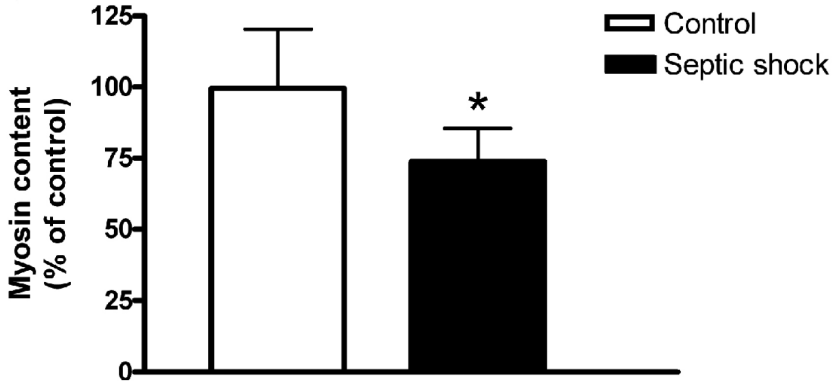

Figure 1 Myosin content in myotubes that were either exposed to plasma from controls (n=12) or plasma from patients with a septic shock (n=21). *$p < 0.01$ vs. controls.

NFkB activity and activation of the ubiquitin-proteasome pathway

We measured NFkB activity as it is a key regulator of the inflammatory response and increased NFkB activity has been associated with activating the ubiquitin-proteasome pathway and inducing muscle atrophy [16]. Figure 2A shows that NFkB activity is significantly increased upon exposure to septic plasma ($p<0.05$).

The E3 ligases, MuRF-1 and MAFBx are key regulating enzymes of the proteolytic ubiquitin-proteasome pathway [17]. The mRNA levels of MAFbx and MuRF-1 were significantly higher in myotubes exposed to septic plasma (Figure 2B).

Finally, exposure to septic shock plasma increased ubiquitination of myosin ($p<0.05$, Figure 2A).

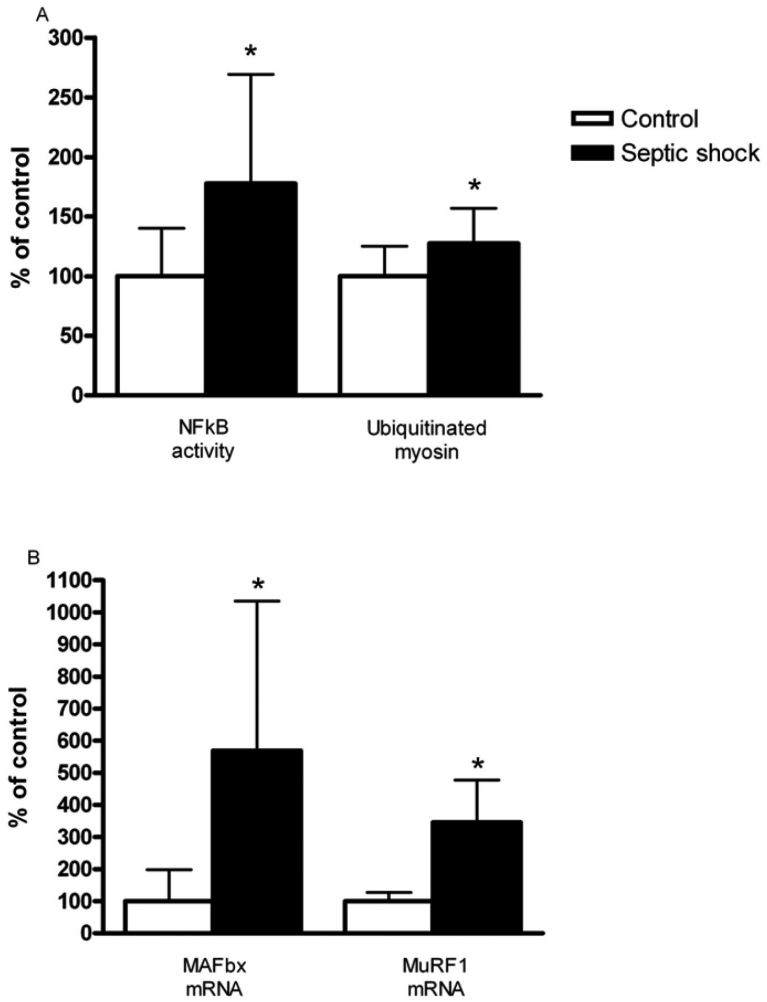

Figure 2 A) NFkB activity in myotubes that were exposed for 1 hour to either plasma from controls (n=6) or to plasma from patients with septic shock (n=6) and ubiquitinated myosin per total myosin levels in myotubes after incubation for 24 hours with either plasma from controls (n=12) or plasma from patients with septic shock (n=12). *$p < 0.05$ vs. controls. B) MAFbx and MuRF-1 expression in myotubes after incubation for 24 hours with either plasma from controls (n=5) or plasma from patients with septic shock (n=14). *$p < 0.05$ vs. controls.

Effects of prolonged septic shock

Because the current data demonstrate that plasma from patients with septic shock induces atrophy at ICU admission, we performed additional experiments to follow this atrophic response in the course of ICU stay.

Myosin content

Figure 3 shows that plasma of patients with septic shock induced the strongest atrophic response at the first day of ICU admission. Exposure of myotubes to plasma obtained at day 2 and 5 after ICU admission also resulted in lower reduced myosin content compared to control, but these differences were borderline statistically significant (p=0.08 and p =0.05 respectively). Myosin content in myotubes that were exposed to plasma obtained after 7 days of ICU care was not significantly different from control (p=0.28). Myosin content at day 7 was significantly higher than at day 0 (p<0.01).

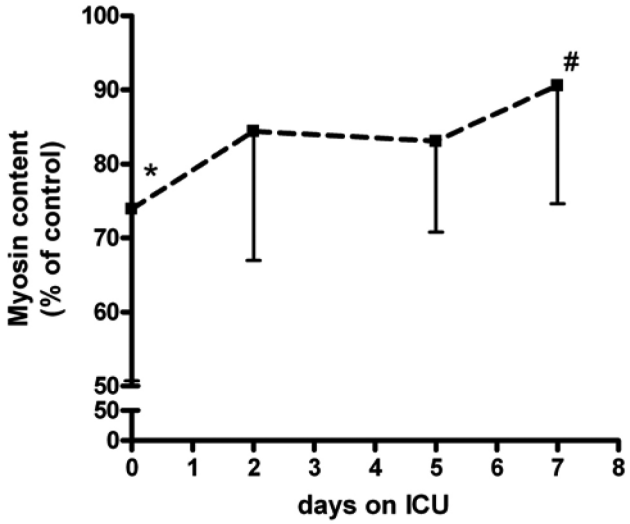

Figure 3 Myosin content in myotubes that were exposed for 24 hours to plasma from healthy controls (n=12) and plasma derived from patients with septic shock (n=9) at subsequent time points during their stay on ICU. *p < 0.05 vs. controls. #p < 0.05 vs. day 0.

E3-ligase expression

MAFbx expression was highest in myotubes that were exposed to plasma taken at the first day of ICU admission (Figure 4). Plasma taken beyond that day did not significantly enhance MAFbx expression. MAFbx expression at day 5 and 7 were significantly lower than at day 0. In contrast, MuRF-1 expression was enhanced in myotubes by plasma obtained up to day 7 after ICU admission (Figure 4).

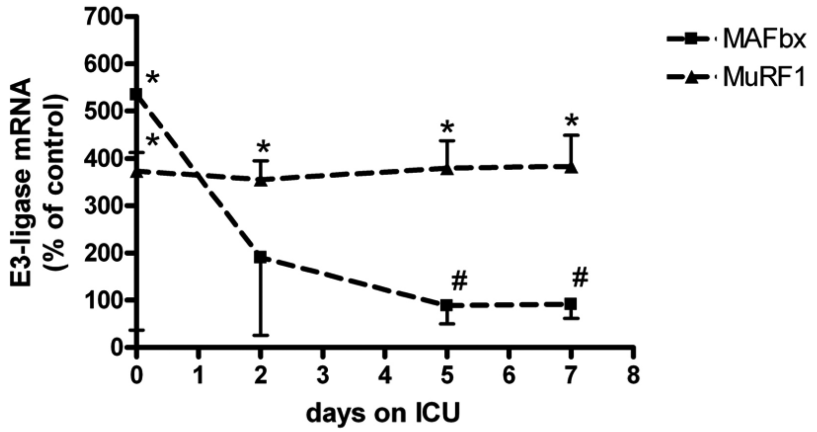

Figure 4 MAFbx and MuRF-1 expression in myotubes that were exposed for 24 hours to plasma from healthy controls (n=5) and plasma derived from patients with septic shock (n=9) at subsequent time points during their stay on ICU. *p < 0.01 vs. controls. #p < 0.05 vs. day 0.

Cytokines

Since cytokines are known to induce proteolysis in muscle cells, we measured IL-1β, IL-6, IFN-γ and TNF-α levels in the plasma samples that were used in the experiments described above. Upon admission to the ICU, IL-6 levels were ~50-fold higher in plasma from patients with septic shock compared to controls (p<0.001, Figure 5). Although IL-6 levels decreased rapidly during hospitalization, levels at day 2, 5 and 7 were still ~10-fold higher than in controls (p=0.05, p=0.09 and p=0.10 respectively), but significantly lower than at day 0 (p<0.01). Correlation statistics showed a significant negative association between myosin content and plasma IL-6 levels (p=0.001, Figure 6).

IFN-γ, TNF-α and IL-1β levels were below the detection limit (resp. 8, 3 and 8 pg/ml) for all controls and for 7 of 9 patients with septic shock.

As these data suggest a causative role of plasma IL-6 in inducing muscle atrophy during septic shock, we performed two additional experiments. First we examined the effect of blocking IL-6 in septic shock plasma on the atrophic response of skeletal myotubes. Figure 7A demonstrates that addition of anti-IL-6 (100ng/ml) to plasma from septic shock patients results in ~25% higher myosin content in skeletal myotubes. In addition, we examined the effect of elevated IL-6 concentration in plasma from controls on the atrophic response of skeletal myotubes. Figure 7B shows that addition of physiological IL-6 concentrations (i.e. 200 and 600 pg/ml, the mean IL-6 plasma levels in septic patients at respectively day 2 and day 0) and supra-physiological (50 ng/ml) concentrations of IL-6 to plasma from controls did not induce an atrophic response in skeletal myotubes.

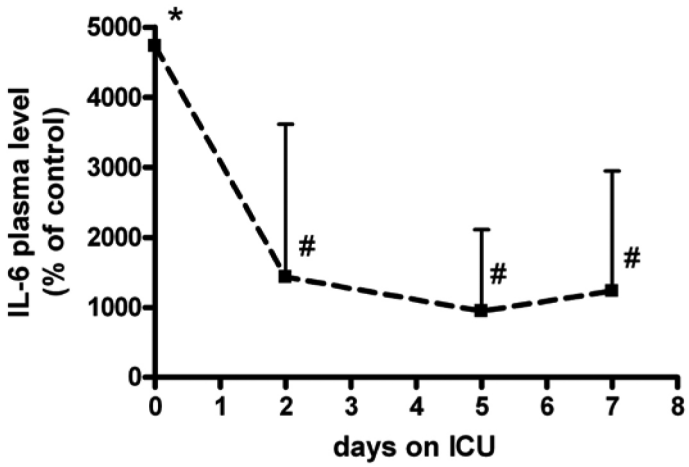

Figure 5 IL-6 levels in plasma from controls (n=9) and from patients with septic shock (n=9) taken within 24 hours of ICU admission and two, five and seven days later. *p < 0.01 vs. controls. #p < 0.05 vs. day 0.

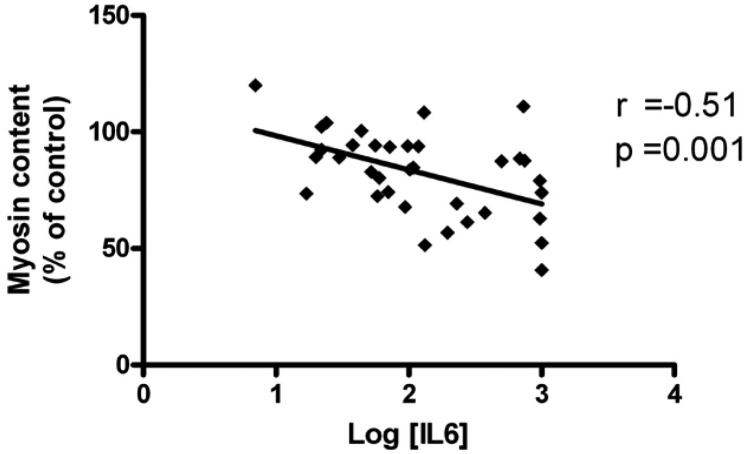

Figure 6 Correlation between plasma IL-6 levels and myosin content in myotubes exposed to plasma from septic shock patients.

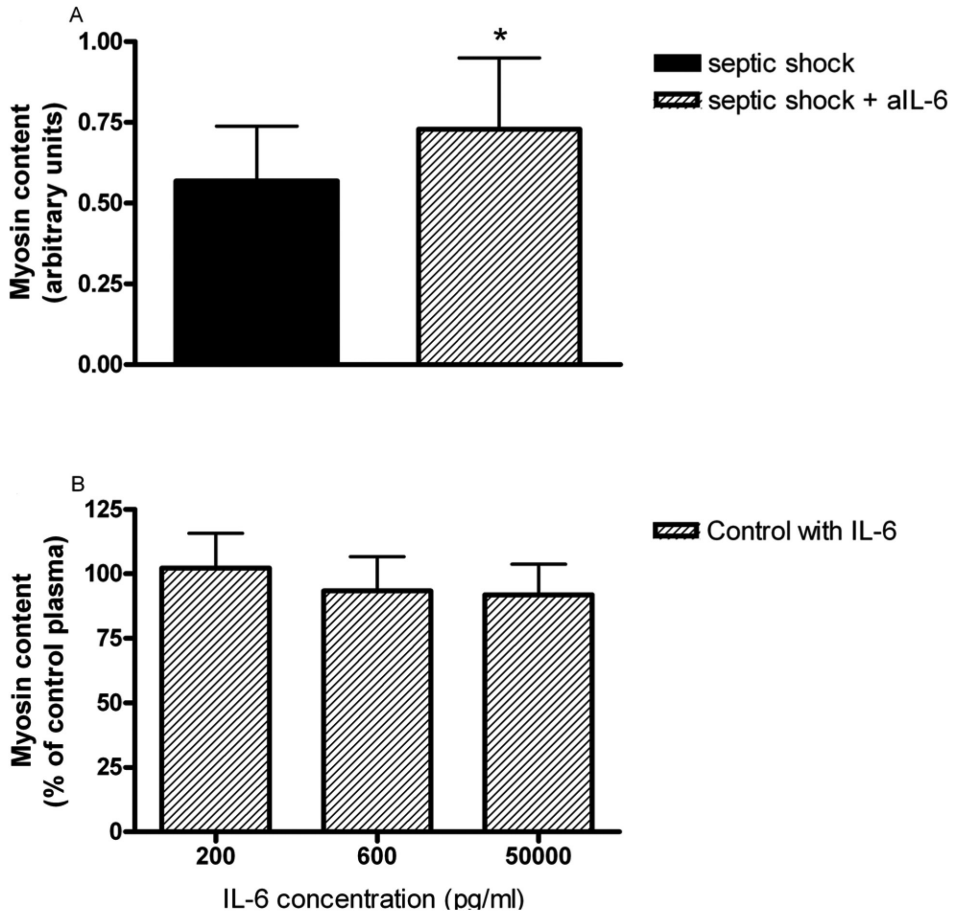

Figure 7 A) Myosin content in myotubes that were exposed to plasma from septic shock patients (n=10), with and without addition of anti-IL6 (100 ng/ml). * $p<0.05$. B) Myosin content in myotubes that were exposed to plasma from controls (n=10) with addition of IL-6 in different concentrations.

DISCUSSION

The present study is the first to demonstrate that (1) plasma from patients with septic shock induces loss of myosin and activation of the ubiquitin-proteasome pathway and (2) that the atrophic response initiated by plasma from septic shock patients is most severe immediately upon arrival at the ICU and decreases during subsequent days and (3) IL-6 plays a prominent role in inducing the atrophic response.

Study limitations

The use of cultured C2C12 skeletal myotubes in the current study is highly appropriate to specifically study the contribution of plasma ligands in muscle wasting, since the use of non-diseased muscle tissue virtually excludes any contribution of intrinsic muscle abnormalities. Other in vitro models have been used previously by others, including dissected muscle bundles [8,18]. A disadvantage of this latter model is the absence of muscular microcirculation. This induces oxidative stress and limits the supply of ligands to the muscle bundle [19,20]. This may explain why incubation with *non-septic* human plasma also increased protein degradation in these models [8]. Therefore, we considered that model not suitable for the current research questions.

C2C12 skeletal myotubes reach a considerable degree of differentiation, as indicated by expression of fast twitch skeletal muscle troponin T, alpha-actin and tropomyosin [21]. Permeabilized myotubes generate force when perfused with calcium solutions, with a similar calcium sensitivity of force generation compared to mature skeletal muscle fibers [21]. Nevertheless, data from our own lab [11] and others [22,23] demonstrated differences in some physiological processes compared to mature skeletal muscle, such as intracellular calcium handling. Accordingly, C2C12 cultured myotubes are suitable for addressing specific research questions, but limitations should be recognized.

Induction of muscle atrophy in septic shock

Sepsis affects skeletal muscle physiology at different stages of excitation-contraction coupling, including contractile protein function, muscle protein content (atrophy), membrane excitability and mitochondrial function [24]. We found that myosin content is reduced by ~25% after exposure of muscle to plasma from patients with septic shock. These data are in line with previous studies on rodents, showing a rapid loss of muscle mass upon induction of sepsis [6,25,26]. The triggers that activate muscle proteolysis in early septic shock are largely unknown. Observational studies have proposed several risk factors, including cytokines, corticosteroids, hyperglycemia and immobilization [1]. Yet, as their effects intermingle in vivo, it is very difficult to establish which of these risk factors do play a role in skeletal muscle wasting in critically ill patients. By specifically studying the potency of plasma to induce muscle wasting, the current study demonstrates that plasma ligands play a prominent role in inducing muscle proteolysis in patients with septic shock. A previous study found that serum of critically ill patients affect membrane excitability and the excitation-contraction coupling process of isolated muscle fibers [27]. Altogether these findings indicate that circulating factors contribute to the development of muscle weakness in critically ill patients.

We demonstrated that the atrophic response to plasma obtained beyond the day of ICU admission was less prominent, but still present. These findings underscore the necessity of early interventions in the prevention of muscle atrophy in septic shock patients. Since the set-up in the current study specifically addresses the effect of plasma ligands, we do not exclude that other factors, such as immobilization [28] and production of inflammatory mediators by the muscle itself [29,30] also contribute to muscle wasting in septic patients, in particular during prolonged ICU stay.

Circulating ligands

An important question is which plasma factors in septic shock patients initiate muscle atrophy. First, inflammatory cytokines such as IL-6, TNF-α, IL-1-β and IFN-γ are often implicated in muscle wasting diseases. In our study, the latter three cytokines were below the detection limit in all healthy subjects and majority of the septic shock patients, suggesting that these were not a major factor in the development of atrophy in our model. Plasma IL-6 levels were elevated in patients with septic shock and plasma levels significantly correlated with the severity of myosin loss. Moreover, blocking IL-6 in plasma from septic shock patients diminished the atrophic response in skeletal myotubes. These data indicate a prominent role of IL-6 in inducing muscle atrophy during septic shock. Noteworthy, addition of IL-6 to plasma from controls did not induce atrophy of skeletal myotubes, even when a supra-physiological IL-6 concentration of 50 ng/ml was applied. Thus, while IL-6 in plasma from septic shock patients is important to induce severe muscle atrophy, other plasma factors seem to be needed as well. Secondly, hyperglycemia has been associated with muscle wasting in critically ill patients [31] and hyperglycemia induces protein degradation in cultured muscle [32]. Yet, patients in the current study were normoglycemic (average glucose level 6.8 ± 0.9 mM), as strict glucose control is part of our routine clinical care. Accordingly, it is unlikely that hyperglycemia did directly contribute to muscle wasting in our model. Thirdly, neuromuscular blocking agents have been associated with the development of muscle atrophy [33], although this has been challenged in recent clinical studies [34]. Nevertheless, the last bolus of rocuronium was administered more than 4 hours before blood withdrawal, ruling out an effect of rocuronium in muscle wasting in our study. Fourthly, high dose of corticosteroids have been associated with skeletal muscle wasting [35]. In the current study, 13 out of 21 patients received low dose (maximal one bolus of 100 mg i.v.) hydrocortisone prior to blood withdrawal. No significant difference in atrophy response at day 1 was observed between patients that had received hydrocortisone and steroid naive patients (Figure 3C). Moreover, at the time more patients had received hydrocortisone (day 2 to 7) the atrophic response

was lower than on day 1. Plasma cortisol concentrations in five of the studied septic shock patients were above normal levels (150-700nM). Yet, these plasma samples provoked similar reductions of myosin in myotubes as plasma samples with normal cortisol levels (data not shown). Finally, metabolic acidosis is known to induce muscle proteolysis by a glucocorticoid-dependent mechanism [36]. Although plasma pH in most patients was acidic, there was no significant relation between plasma pH and myosin concentration (data not shown).

Intracellular mechanisms

The main focus of this study was to investigate whether myosin loss is triggered by plasma from patients with septic shock, but we also studied activation of proteolysis in these skeletal myotubes. The ubiquitin-proteasome pathway is the main proteolytic system in eukaryotic cells and controls both protein quality and quantity [37]. During the course of this pathway proteins are linked to a chain of ubiquitin molecules under regulation of E3-ligases such as MuRF-1 and MAFBx and subsequently recognized and degraded by the proteasome. Recent evidence from our lab indicates that myosin degradation follows this pathway, as proteasome inhibition restores myosin content and muscle function in animal models for respiratory muscle weakness [15]. Moreover, components of the ubiquitin-proteasome pathway are up-regulated in skeletal muscle of septic shock patients [38,39]. The current study adds to these earlier observations as we show that plasma from septic shock patients increases ubiquitinated myosin levels and activates MuRF-1 and MAFBx in skeletal myotubes. MuRF-1 has been shown to specifically ubiquitinate myosin, thereby promoting myosin degradation [40,41]. MuRF-1 expression is under control of the transcription factor NFkB [42]. Indeed, exposure to patient plasma increases the activity of NFkB in myotubes within 1 hour. MAFbx expression occurs independent from NFkB activity, but is also associated with loss of muscle proteins [43]. Noticeably, in mice overexpression of circulating IL-6 enhances MAFbx mRNA and induces loss of muscle mass [44]. In line with that study, we found that MAFbx expression diminished after day 2 on the ICU and followed a similar trend as IL-6, which in turn is inversely related to myosin content. Therefore, these data further support the notion that IL-6 is involved in the initiation of skeletal muscle atrophy in septic shock patients.

CONCLUSION

The present study demonstrates that plasma from patients with septic shock induces loss of the contractile protein myosin in skeletal myotubes. This atrophic response is most severe to plasma from the early phase of sepsis and is associated with activation of key regulators of proteolysis. IL-6 may play a role in the early development of muscle atrophy in septic shock patients.

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Chapter 4

Toll-like Receptor 4 signaling in Ventilator-Induced Diaphragm Atrophy

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ABSTRACT

Background Mechanical ventilation induces diaphragm muscle atrophy, which plays a key role in difficult weaning from mechanical ventilation. The signaling pathways involved in ventilator-induced diaphragm atrophy are poorly understood. The current study investigated the role of toll-like receptor 4 signaling in the development of ventilator-induced diaphragm atrophy.

Methods Unventilated animals were selected for control: wild-type (n=6) and toll-like receptor 4 deficient mice (n=6). Mechanical ventilation (8 hours): wild-type (n=8) and toll-like receptor 4 deficient (n=7) mice.
Myosin heavy chain content, pro-inflammatory cytokines, proteolytic activity of the ubiquitin-proteasome pathway, caspase-3 activity and autophagy were measured in the diaphragm.

Results Mechanical ventilation reduced myosin content by ~50% in diaphragm of wild-type mice ($p < 0.05$). In contrast, ventilation of toll-like receptor 4 deficient mice did not significantly affect diaphragm myosin content. Likewise, mechanical ventilation significantly increased interleukin-6 and keratinocyte-derived chemokine in the diaphragm of wild-type mice, but not in ventilated toll-like receptor 4 deficient mice. Mechanical ventilation increased diaphragmatic Muscle Atrophy Factor box transcription in both wild-type and toll-like receptor 4 deficient mice. Other components of the ubiquitin-proteasome pathway and caspase-3 activity were not affected by ventilation of either wild-type mice or toll-like receptor 4 deficient mice. Mechanical ventilation induced autophagy in diaphragm of ventilated wild-type mice, but not toll-like receptor 4 deficient mice.

Conclusion Toll-like receptor 4 signaling plays an important role in the development of ventilator-induced diaphragm atrophy, most likely through increased expression of cytokines and activation of lysosomal autophagy.

INTRODUCTION

Invasive mechanical ventilation is a life saving intervention in patients with acute respiratory failure. However, it is well known that mechanical ventilation comes with important adverse events. For instance, studies in both rodents and humans have shown that controlled mechanical ventilation results in atrophy and weakness of the respiratory muscles [1,2]. This is an important clinical problem, as inspiratory muscle weakness plays a prominent role in patients difficult to wean from mechanical ventilation [3].

The molecular pathways involved in the development of respiratory muscle atrophy during mechanical ventilation are incompletely understood. Increased muscle protein breakdown as well as reduced muscle protein synthesis have been associated with diaphragm atrophy induced by mechanical ventilation [1-5]. The upstream pathways that induce this imbalance in muscle protein turnover are currently unclear. Cytokines are well-known modulators of muscle protein turnover [6] and are involved in the development of respiratory muscle atrophy under inflammatory conditions [7]. In the last decade it has been established that mechanical ventilation itself is able to provoke a local and systemic inflammatory response [8,9]. Yet, whether mechanical ventilation induces an inflammatory response in the diaphragm has never been investigated. Toll-like Receptors (TLR) are crucial receptors in the initiation of an inflammatory response. Different types of TLRs recognize specific ligands, which include microbial components, but also proteins released from damaged tissue (‘alarmins’) (For review [10]). Recent research demonstrated that the pulmonary inflammatory response to mechanical ventilation partly depends on TLR4 signaling [11,12]. TLR4 is also expressed on muscle tissue, including the diaphragm [13]. Accordingly, administration of the TLR4 specific ligand lipopolysaccharide to rodents elicits an up regulation of pro-inflammatory genes in the diaphragm [14] and reduces diaphragm strength [15]. Whether TLR4 plays a role in ventilator-induced diaphragm atrophy is unknown, but of potential interest as TLR4 antagonists are available for use in humans.

Therefore, the first aim of the present study was to investigate whether mechanical ventilation-induced diaphragm atrophy is associated with an inflammatory response in the diaphragm. The second aim was to establish whether ventilator-induced diaphragm atrophy and inflammation depend on TLR4 signaling.

MATERIALS & METHODS

Animals

Experiments were carried out in male C57BL/6 mice (n=14) aged 21 ± 0.7 weeks, bodyweight 27 ± 0.5 gram (Charles River, Sulzfeld, Germany) and male TLR4 knockout mice (n=13) aged 20 ± 0.6 weeks, weighing 31 ± 0.7 gram (C57BL/6 background). All TLR4 knockout mice were extensively backcrossed (at least 10 times) and were a gift from Professor Shizuo Akira, M.D., Ph.D. (Osaka University, Osaka, Japan). Animals were fed ad libitum.

To determine the role of TLR4 in ventilator-induced diaphragm atrophy, four groups of mice were studied: control wild-type (cWT; n=6), mechanically ventilated wild-type (mvWT; n=8), control TLR4 knockout (cTLR4 KO; n=6) and mechanically ventilated TLR4 knockout (mvTLR KO; n=7).

All experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care.

Controlled mechanical ventilation

Mice selected for ventilation were anesthetized and mechanically ventilated as described previously with minor modifications [11]. Briefly, mice were ventilated with a tidal volume of 8 ml/kg bodyweight, respiratory rate of 170/min, positive end-expiratory pressure of 1.5 cm H_2O and inspired oxygen fraction of 0.45.

A sterile catheter was inserted in the carotid artery for continuous blood pressure monitoring. The cWT and cTLR4 KO mice were anesthetized and sacrificed without being mechanically ventilated as described previously [9]. Previous investigations from our lab have established that this experimental setting is free from contamination with lipopolysaccharide [11].

Tissue collection

After 8 hours of mechanical ventilation (mechanical ventilation groups) or immediately after anesthesia (controls), mice were exsanguinated and a combined thoracotomy and laparotomy was performed. Left and right hemidiaphragm tissue was rinsed with the left part quickly frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis and the right hemidiaphragm submersed in cooled Krebs solution at pH 7.4 for single fiber isolation.

Cytokines in diaphragm and plasma

Levels of tumor necrosis factor α**,** interleukin (IL)-1α, IL-1β, IL-6 and keratinocyte-derived chemokine (KC) in the diaphragm and IL-1β, IL-6 and KC in plasma were

analyzed by enzyme-linked-immunosorbent assay as published previously [11]. To determine cytokine levels in the diaphragm, the muscle was homogenized in 100 volumes of ice-cold buffer, pH 7.2 (10 mM Tris/Maleate, 3 mM EGTA, 275 mM sucrose, 0,1 mM dithiothreitol, 2 mg/ml Leupeptine, 2 mg/ml Aprotinine, 10 mg/L Pepstatine A, 0,57 mM phenylmethylsulphonylfluoride) three cycles of freezing and thawing and centrifuged at 17.000 G at 4°C for 30 minutes. Lower detection limits were 40 pg/ml for IL-1α and IL-1β; 32 pg/ml for tumor necrosis factor α; 160 pg/ml for IL-6 and for KC.

Single fiber myosin heavy chain content

As described previously, content of myosin heavy chain was determined in isolated single fibers [16] with minor modifications. In short, after isolation, the length of a single fiber was measured by making a microscopic image on top of a metal raster. The single fiber length was analyzed using an image analysis system (ImageJ version 1.42, U.S. National Institutes of Health, Bethesda, MD). Subsequently, fibers were analyzed for myosin heavy-chain content by SDS–polyacrylamide gel electrophoresis.

Ubiquitinated myosin heavy chain content

Ubiquitinated myosin and total myosin were determined as described before [17]. Diaphragm samples were homogenized in 100 volumes of ice-cold buffer containing 20mM Tris-HCl (pH 7.4), 20 mM EGTA, 1mM dithiothreitol, 0,5% SDS and protease inhibitor cocktail (Sigma, Saint Louis, MO), boiled and centrifuged. Soluble proteins were subjected to routine Western blotting. Anti-ubiquitin antibodies (PW8805, Biomol, Plymouth Meeting, PA) and anti-myosine (my-32, Sigma) were used to stain ubiquitinated myosin and total myosin, i.e. both ubiquitinated and not ubiquitinated myosin. Secondary goat antimouse-polyvalent immunoglobulins peroxidase conjugate (A0412, Sigma) and ECL kit (GE healthcare, Buckinghamshire, United Kingdom) were applied for detection and analysis of ubiquitinated protein bands (optical densitometry software from Syngene, Cambridge, United Kingdom). Goat antimouse IRDye 800CW (LI-COR, Lincoln, NE) and subsequent Odyssey scan and Odyssey application software version 2.1 (LI-COR) were used for analysis of the myosin signal. For each lane the ratio of optical densities of ubiquitinated myosin per total myosin was calculated.

Ubiquitin-proteasome pathway and caspase-3 activity

To assess involvement of proteolysis we measured 20S proteasome proteolytic activity and caspase-3 activity as described previously [17]. The proteolytic activity of isolated 20S proteasomes was determined by measuring the generation of the fluorogenic cleavage product methylcoumarylamide from the

fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY) by spectrophotometry.
The caspase-3 activity was determined by measuring the generation of the fluorogenic cleavage product methylcoumarylamide from the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by spectrophotometry. In addition, we measured the presence of 14kD actin, a specific breakdown product of caspase-3 [18] by Western blotting and anti-actin antibody (A2066, Sigma).
To establish whether regulation of the ubiquitin-proteasome pathway was modulated, Muscle RING-finger protein-1 (MuRF-1) protein content was assessed by Western blotting using anti-MuRF-1 antibodies (Ab77577, Abcam, Cambridge, United Kingdom) and messenger ribonucleic acid levels of MuRF-1 and Muscle Atrophy Factor box (MAFbx) were determined by quantitative polymerase chain reaction [17]. Levels of MAFbx and MuRF-1 messenger ribonucleic acid were normalized to glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid. Forward and reverse oligonucleotides used were as following:
MAFbx, 5'-GACTGGACTTCTCGACTGCC-3' and 5'-TCAGCCTCTGCATGATGTTC-3', MuRF-1, 5'-CAACCTGTGCCGCAAGTG-3' and 5'-CAACCTCGTGCCTACAAGA TG-3'; Glyceraldehyde-3-phosphate dehydrogenase, 5'-TGATGGGTGTGA ACCACGAG-3' and 5'-GGGCCATCCACAGTCTTCTG -3'.

Induction of autophagy
To study the role of lysosomal autophagy, the content of autophagy marker Light Chain 3B-II [19] (LC3B-II) was measured using standard Western blotting as described previously [20] using a specific antibody against LC3B 2775 (Cell signaling Technology, Danvers, MA). Optical density of LC3B-II bands on blot were quantified using Odyssey scan and Odyssey application software version 2.1 (LI-COR).

Statistical Analysis
A two-sided unpaired Student t-test was performed to evaluate the statistical significance of differences for myosin heavy chain and LC3B-II between cWT and mvWT animals, between cTLR4 KO and mvTLR4 KO animals.
Differences between the groups regarding cytokines, MAFbx, MuRF-1 messenger ribonucleic acid, MuRF-1 and actin protein, caspase-3 activity were analyzed with one-way ANOVA. Student-Newman-Keuls post hoc testing was used to test the probability level of differences between cWT and mvWT animals, between cTLR4 KO and mvTLR4 KO animals, between cTLR4 KO and cWT and between mvWT and mvTLR4 KO animals. For statistical analysis of cytokine measurements the value of the detection limit was used for samples that did not reach the detection limit.

Graphpad prism was used to conduct statistical analysis (Graphpad Software Inc., San Diego, CA). A probability level of $p<0.05$ was considered significant. All data, except plasma cytokines, are presented as mean ± SE. Plasma cytokines were presented as median (interquartile range, IQR) and mean.

RESULTS

Although initially mean arterial blood pressure decreased in both groups, most likely resulting from anesthesia (Figure 1), hemodynamics stabilized thereafter. Bloodgas analysis after 8 hours of mechanical ventilation showed that PaO_2, $PaCO_2$, bicarbonate and A-a gradient were not significantly different between both ventilated groups (Table 1). mvWT mice were mildly acidotic after 8 hours of mechanical ventilation.

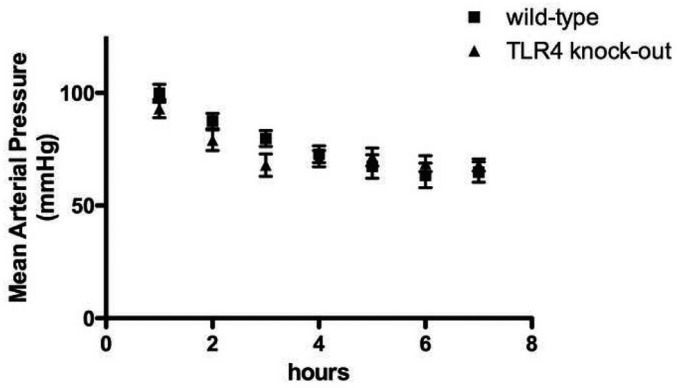

Figure 1 Mean Arterial Pressure during mechanical ventilation.

	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	HCO_3 (mmol/L)	A-a gradient (mmHg)	BE (mEq/L)
WT	7.31 ± 0.02*	203 ± 23	26 ± 3.6	16 ± 1	79 ± 21	−11.5 ± 1,4
TLR4 KO	7.40 ± 0.02	220 ± 20	24 ± 1.4	18 ± 1	64 ± 20	−8.0 ± 1,1

Table 1 Arterial bloodgas and alveolar-arterial gradient after 8 hours of mechanical ventilation * $p < 0.05$ vs. ventilated TLR4 KO; Values are mean ± SEM. BE = base excess; $PaCO_2$ = arterial carbon dioxide tension; PaO_2 = arterial oxygen tension; A-a gradient = Alveolar arterial gradient.

Cytokines in diaphragm

Mechanical ventilation significantly increased levels of IL-6 (cWT 230 ± 26 pg/mg vs. mvWT 356 ± 37 pg/mg) by ~55% (Figure 2A, $p < 0.02$) and KC (cWT 87 ± 28 pg/mg vs. mvWT 183 ± 22 pg/mg) by ~110% (Figure 2B, $p < 0.002$) in the diaphragm of WT mice. Although levels of IL-1β (cWT 49 ± 11 pg/mg vs. mvWT 67 ± 10 pg/mg) were ~37% higher in the diaphragm of mechanically ventilated WT mice compared to unventilated WT mice this difference did not reach statistical significance (Figure 2C). A similar trend regarding elevation of cytokine levels was observed in the diaphragm of mvTLR4 KO mice. Yet, knock out of TLR4 clearly dampened the inflammatory response in the diaphragm upon mechanical ventilation, since none of the cytokine protein levels in the diaphragm were significantly different between ventilated and unventilated TLR4 KO mice. KC and IL-6 levels in the diaphragm were significantly lower in ventilated TLR4 KO than in ventilated WT mice ($p < 0.05$). Diaphragmatic levels of IL-1α and tumor necrosis factor α were not affected after 8 hours of mechanical ventilation in either WT or TLR4 KO mice. Deficiency of TLR4 did not affect diaphragm muscle cytokine levels in unventilated animals.

Cytokines in plasma

In ventilated WT mice, plasma levels of KC were significantly increased compared to unventilated mice (below detection limit for control WT and median 5060 [1440, 10910] pg/ml, mean 5952 pg/ml for ventilated WT; $p < 0.02$). Although plasma levels of IL-1β (below detection limit for control WT and median 40 [40, 252] pg/ml, mean 125 pg/ml for ventilated WT) and IL-6 (below detection limit for control WT and median 4130 [1295, 15575] pg/ml, mean 7574 pg/ml for ventilated WT) were higher in ventilated mice than in control mice, these differences did not reach statistical significance. Similarly, but also statistically not significant, mechanical ventilation of TLR4 KO mice led to elevated plasma levels of KC (below detection limit for control TLR4 KO and median 2020 [1090, 2685] pg/ml, mean 1914 pg/ml for ventilated TLR4 KO) and IL-6 (below detection limit for control TLR4 KO and median 2810 [985, 3505] pg/ml, mean 2358 pg/ml for ventilated TLR4 KO). Plasma IL-1β was not different between control and ventilated TLR4 KO (both TLR4 KO groups were below detection limit). Plasma KC levels, but not IL-6 and IL-1β, were significantly higher in mvWT compared to mvTLR4 KO mice ($p < 0.05$).

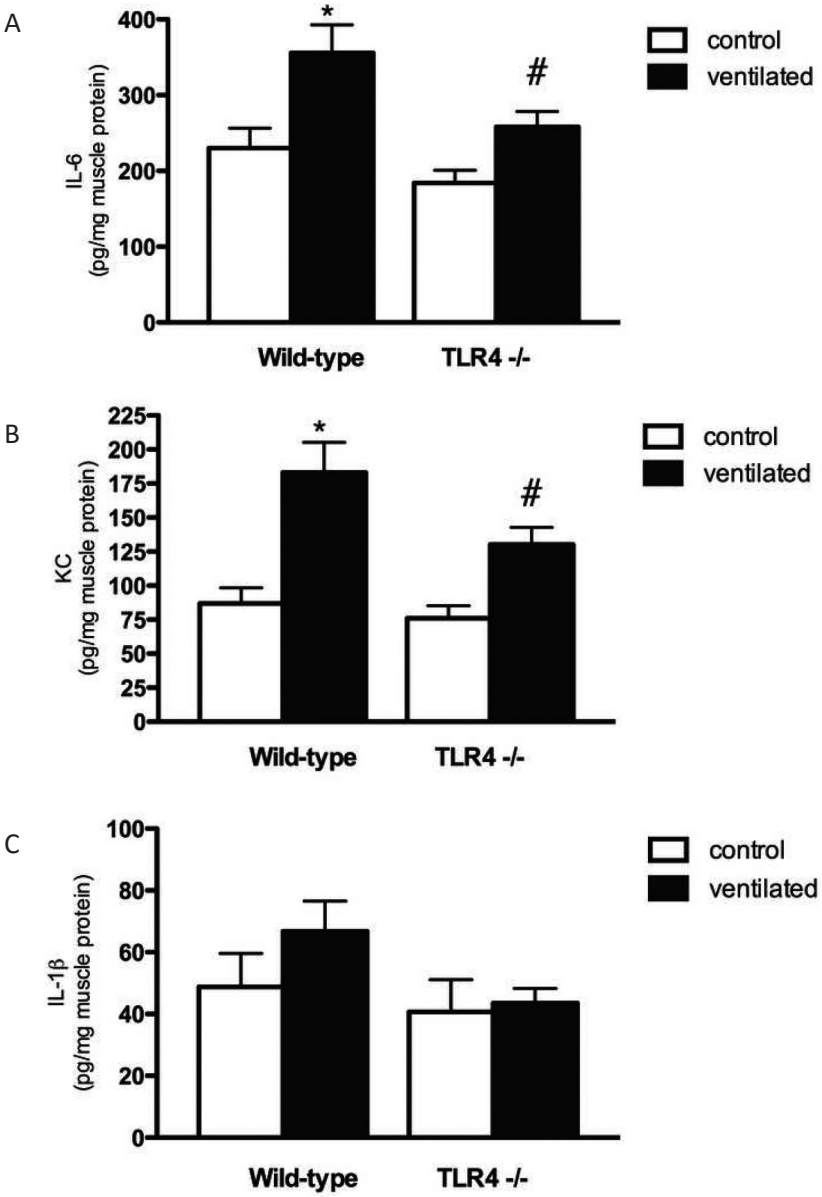

Figure 2 A) IL-6 cytokine level in diaphragm homogenates. * $p < 0.02$ vs. control WT; # $p < 0.05$ vs. ventilated WT. B) KC cytokine level in diaphragm homogenates. * $p < 0.002$ vs. control WT; # $p < 0.05$ vs. ventilated WT. C) IL-1β cytokine level in diaphragm homogenates.

Myosin heavy chain content

In 8 hours ventilated WT mice myosin heavy chain content in diaphragm muscle single fibers was significantly reduced by ~50 % (Figure 3; $p < 0.05$). In contrast, myosin heavy chain content in diaphragm fibers from TLR4 KO mice was not significantly reduced by mechanical ventilation. Myosin heavy chain content was not different between unventilated WT and TLR4 KO mice, neither between ventilated WT and ventilated TLR4 KO mice.

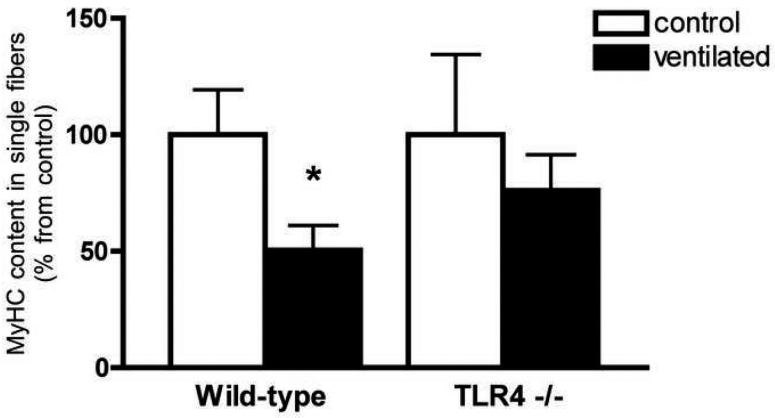

Figure 3 Myosin heavy chain content in diaphragm single fibers from control WT and TLR4 KO, and ventilated WT and TLR4 KO mice. * $p < 0.05$ vs. control WT. Data are represented as % from cWT for mvWT and as % from cTLR4 KO for mvTLR4 KO.

Ubiquitin-proteasome pathway

Mechanical ventilation significantly enhanced transcription of MAFbx in both wild-type and TLR4 KO diaphragm ($p < 0.001$; Figure 4A). MuRF-1 transcription levels and protein content in the diaphragm were not different after 8 hours of mechanical ventilation of either wild-type or knock-out mice (Figure 4B, 4C and 4D). TLR4-deficiency did not affect MAFbx, nor MuRF-1 expression in unventilated animals (Figure 4 A/B/C).

The ratio of ubiquitinated myosin heavy chain over total myosin heavy chain was not significantly affected by mechanical ventilation nor by TLR4 knockout, and levels between cWT and cTLR4 KO were not different (Figure 5). Proteasome activity in the diaphragm was not affected by mechanical ventilation nor by TLR4 deficiency (Figure 6).

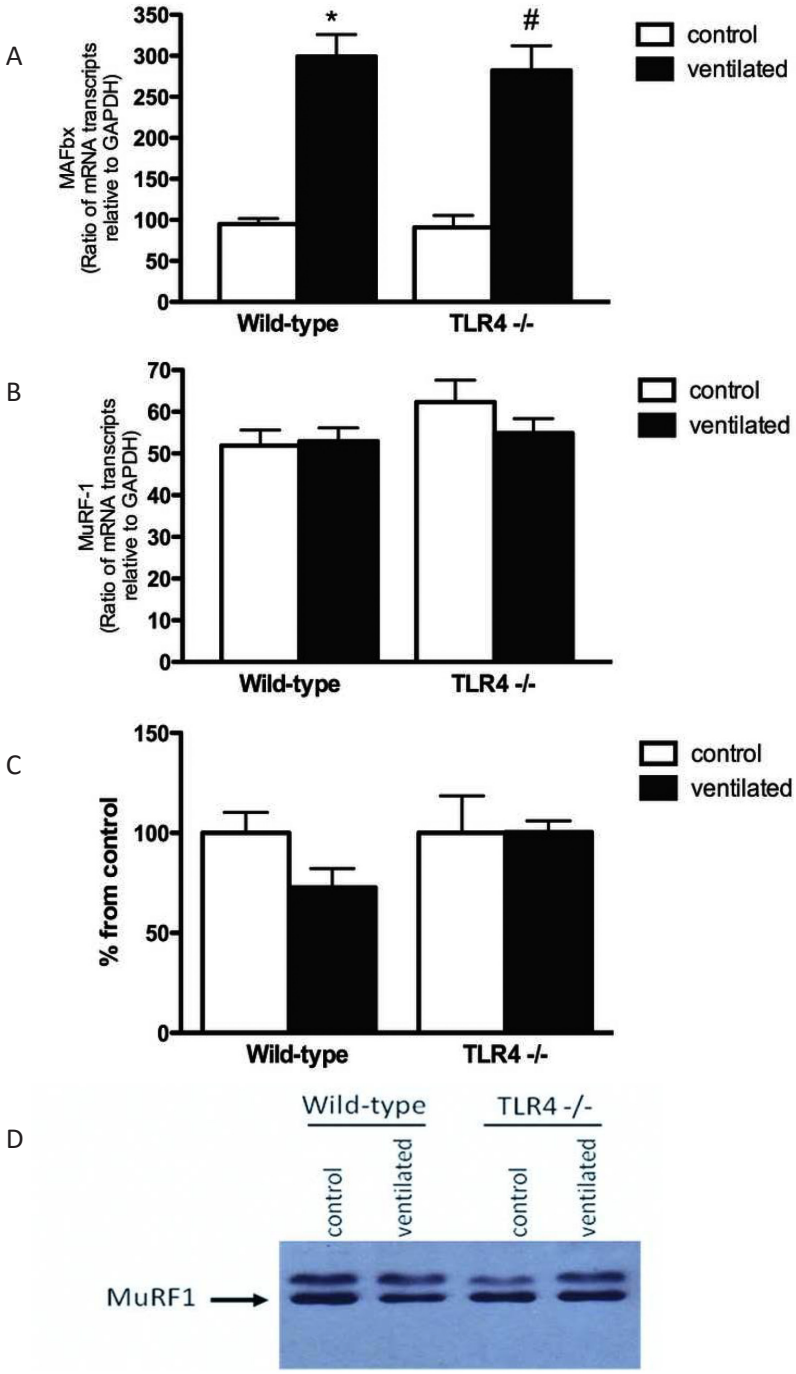

Figure 4 E3-ligase expression levels in the diaphragm of control WT and TLR4 KO and ventilated WT and TLR4 KO mice (ratio of mRNA transcripts relative to GAPDH).
A) MAFbx mRNA levels. * $p < 0.001$ vs. control WT; # $p < 0.001$ vs. control TLR4 KO.
B) MURF-1 mRNA. C) MuRF-1 protein levels. Data are represented as % from cWT for mvWT and as % from cTLR4 KO for mvTLR4 KO. D): representative Western blot stained against MuRF-1.

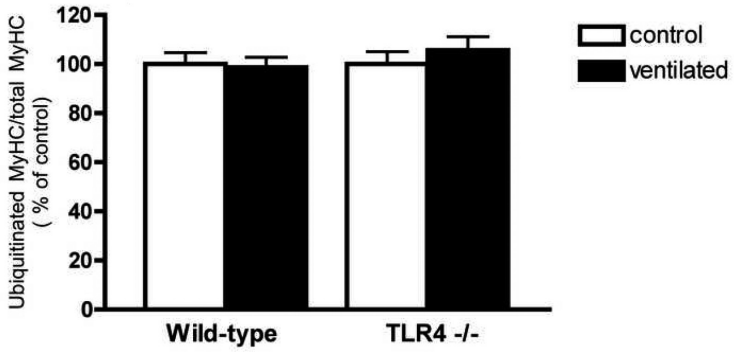

Figure 5 Ubiquitinated myosin levels in diaphragm from control WT and TLR4 KO and ventilated WT and TLR4 KO mice. Data are represented as % from cWT for mvWT and as % from cTLR4 KO for mvTLR4 KO.

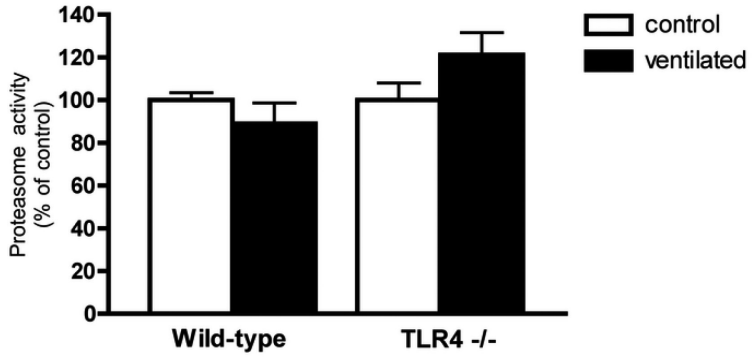

Figure 6 Proteasome activity in the diaphragm of control WT and TLR4 KO, and ventilated WT and TLR4 KO mice. Data are represented as % from cWT for mvWT and as % from cTLR4 KO for mvTLR4 KO.

Caspase-3 activity

No differences in diaphragm caspase-3 activity were observed between groups (Figure 7A). To support this observation we determined levels of 14 kD actin fragments, a product of caspase-3 activation. Indeed, no 14 kD fragment was found in diaphragm homogenates of any group, supporting the absence of caspase-3 activation (Figure 7B).

Autophagy

Content of the autophagy marker LC3B-II was significantly increased by ~31% in diaphragm muscle of 8 hours mechanical ventilated WT mice compared to control WT mice (Figure 8). In contrast, diaphragmatic LC3B-II content in ventilated TLR4 KO mice, was not different from unventilated TLR4 KO. LC3B-II was not different between unventilated WT and TLR4 KO mice, neither between ventilated WT and TLR4 KO mice.

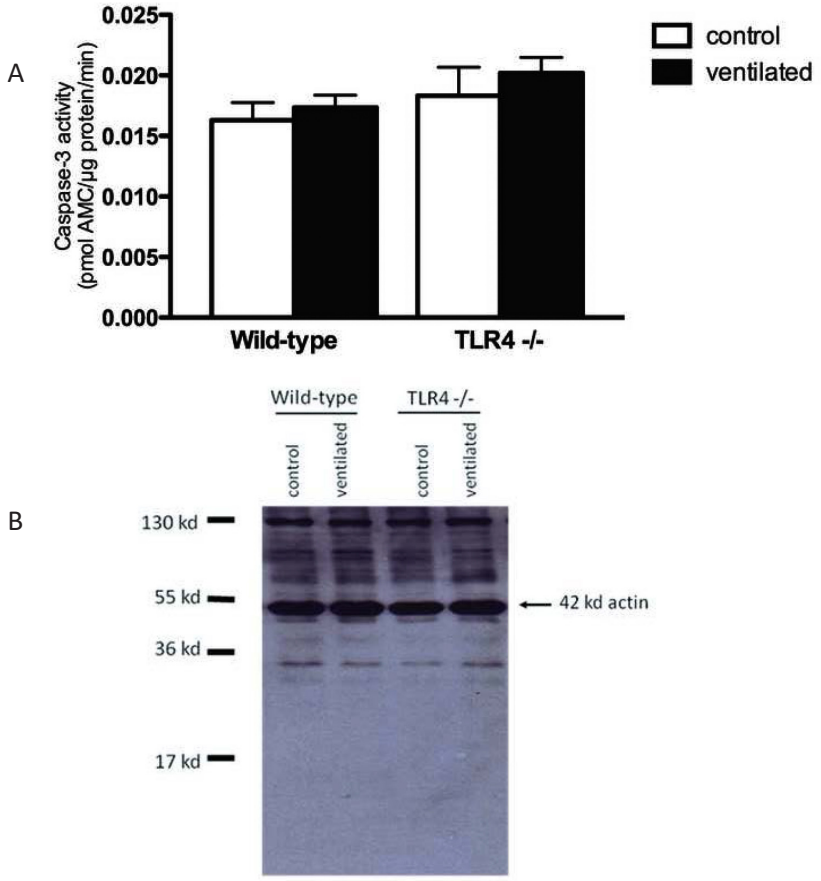

Figure 7 A) Caspase-3 activity in the diaphragm of control WT and TLR4 KO, and ventilated WT and TLR4 KO mice. B) Representative Western blot stained against actin.

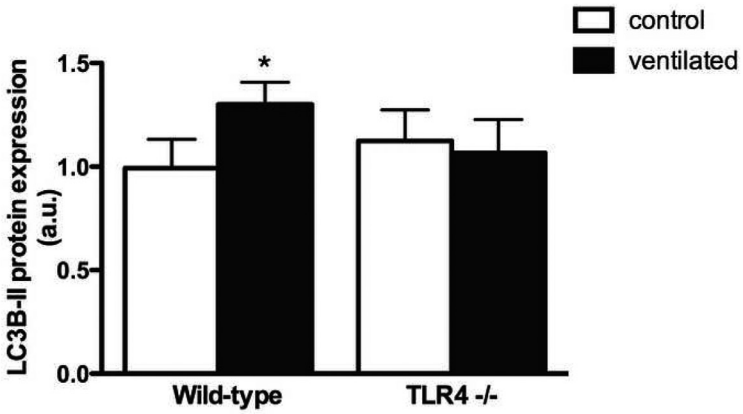

Figure 8 LC3B-II concentration in the diaphragm of control WT and TLR4 KO, and ventilated WT and TLR4 KO mice, data are presented as arbitrary units (a.u.) from Western blot. Mechanical ventilation increases levels of LC3-II in diaphragm of WT mice (* vs. control WT; p = 0.05) and not in TLR4 KO mice.

DISCUSSION

The present study investigated the signaling pathways of ventilator-induced diaphragm muscle atrophy in particular related to TLR4. The main new findings are that: (1) mechanical ventilation-induced diaphragm muscle atrophy is associated with increased expression of cytokines in the diaphragm and (2) TLR4 signaling is involved in myosin loss, the inflammatory response and lysosomal autophagy in the diaphragm during controlled mechanical ventilation. These findings are of potential clinical interest, as diaphragm atrophy plays a prominent role in weaning from mechanical ventilation.

Inflammatory response in diaphragm upon mechanical ventilation

This is the first study to examine the effects of mechanical ventilation on inflammatory responses in the diaphragm. We found that mechanical ventilation increases diaphragm levels of IL-6, KC and to a lesser extend IL-1β. It has previously been proposed that increased expression of cytokines induce skeletal muscle atrophy. For example, overexpression of IL-6 in transgenic mice engenders profound skeletal muscle atrophy, which can be completely blocked by administration of an IL-6 antagonist [21]. Subcutaneous administration of IL-6 also results in skeletal muscle atrophy in rats [22]. Noticeably, dose-response experiments showed diaphragm weight loss at concentrations where no peripheral muscle weight loss was detected, suggesting that the diaphragm is more sensitive to the atrophic effects of IL-6 than peripheral muscles. Furthermore, local infusion of IL-6 into the tibialis anterior in rats causes a preferential loss of myofibrillar proteins, such as myosin [23]. A recent study from our lab showed that IL-6 in plasma of septic patients plays a prominent role in inducing muscle atrophy [24]. Besides IL-6, we found increased levels of KC and IL-1β in the diaphragm of mechanically ventilated mice. As far as we know, no previous studies have investigated the role of KC and IL-1β on muscle atrophy *in vivo*. Nevertheless high circulating levels of IL-1β have been associated with skeletal muscle wasting [25]. Some evidence for a causative role for IL-1β in skeletal muscle wasting comes from *in vitro* studies. For example, exposure of skeletal myotubes to IL-1β during 48 hours results in muscle atrophy [26]. In line with these studies, results from the current study show a relation between increased expression of cytokines and myosin loss in the diaphragm, i.e. in contrast to wild-type mice, mechanical ventilation of TLR4 deficient mice did not result in enhanced cytokine expression nor in reduced myosin content. Although the current data support the concept that the increased levels of cytokines elicited by mechanical ventilation are associated with myosin loss in the diaphragm, we did not investigate a causal relationship. To that end, future studies should examine whether administration of IL-6 antagonists can prevent the induction of diaphragm atrophy during mechanical ventilation.

Role for TLR4 in ventilator induced diaphragm atrophy

The present study shows that knocking out the TLR4 gene prevented increased expression of cytokines and loss of myosin in the diaphragm upon mechanical ventilation. This implicates a role for TLR4 signaling in ventilator-induced diaphragm dysfunction. TLR's are well-known for their role in innate immunity. Each TLR homologue senses a specific set of conserved microbial molecules. The post-receptor signaling of TLR's is very complex (for a detailed review see [27]), but noteworthy is that activation of TLR's eventually results in the release of inflammatory cytokines, necessary to combat infection. Recent discoveries show that TLRs, in particular TLR4, are expressed in skeletal muscle [13,28,29]. Moreover, several studies have demonstrated that stimulation of TLR4 by lipopolysaccharide increases the expression of cytokines, like IL-6, KC, IL-1β and tumor necrosis factor α, in skeletal muscles [13,28,30]. The inflammatory response to lipopolysaccharide administration also occurs in the diaphragm, where it is even more vigorous than in limb muscle [14]. In line with these data, the current study shows that TLR4-deficiency attenuated the upregulation of cytokines in the diaphragm upon mechanical ventilation. Moreover, we found that knocking out the TLR4 gene partially prevented myosin loss in the diaphragm of mechanically ventilated mice. Since myosin plays a central role in muscle contraction, these data indicate that TLR4 signaling is involved in mechanical ventilation-induced diaphragm dysfunction.

Although it was not a main objective of this study, our data provide some additional insight into the down-stream mechanisms by which TLR4 signaling induces loss of myosin. For instance, considering the well established effect of increased expression of cytokines on myosin content as described above, it seems likely that myosin loss in the diaphragm upon mechanically ventilation is caused by TLR4-mediated upregulation of inflammatory cytokines.

A recent publication by Doyle et al. suggested that TLR4 activation might also directly induce myosin loss by the p38 mitogen-activated protein kinase pathway, i.e. independent from actions of cytokines [20]. Myosin loss in that study was provoked by coordinate downstream activation of the ubiquitin-proteasome and autophagy-lysosome pathways. Interestingly, our data indicate that mechanical ventilation activates autophagy in the diaphragm already after 8 hours. This is in line with a recent study in humans, which showed activation of autophagy after prolonged mechanically ventilation [31]. More importantly, our data show that TLR4 plays a prominent role in inducing autophagy during mechanical ventilation, because TLR4 knockout mice did not show upregulation of LC3B-II after 8 hours of mechanical ventilation. Remarkably, our data do not support an important role for the ubiquitin-proteasome pathway in ventilator-induced diaphragm atrophy. First of all, 8 hours of mechanical ventilation did not enhance proteasome activity.

Secondly, reduced total myosin content was not accompanied by increased ubiquitination of myosin. In accordance, expression of the E3-ligase MuRF-1, that is known to ubiquitinate myosin [32], was unaffected by mechanical ventilation. Finally, although mechanical ventilation increased MAFbx expression, the protective effects of TLR4 deficiency on myosin content were independent from MAFbx activation. In contrast, some previous studies showed that diaphragm atrophy was associated with activation of the ubiquitin-proteasome pathway in the diaphragm of mechanical ventilated rats [5,33,34] and brain-dead humans [35]. Yet, those studies do not provide unambiguous evidence that this pathway is responsible for the loss of myosin. In contrast to the current study, ubiquitination of myosin was not specifically studied. Our data indicate that 8 hours of mechanical ventilation does not increase ubiquitination of myosin. Furthermore, attenuation of mechanical ventilation-induced diaphragm atrophy by anti-oxidants occurs independent from elevated MuRF-1 and MAFbx expression [36]. Caspase-3 is known to cleave the contractile protein actin, which may induce release of myosin from the sarcomere [18]. However, data from the present study do not support a role for caspase-3, as its cleaving activity in the diaphragm was not affected by mechanical ventilation and actin fragments could not be detected. In apparent contrast with our study, previous studies have shown enhanced content of activated caspase-3 in the diaphragm of mechanical ventilated animals [37,38] and humans [1]. However, in those studies cleavage activity of caspase-3 itself was not measured nor were concentrations of contractile proteins determined. With respect to those studies, we do therefore not exclude that the ubiquitin-proteasome pathway and caspase-3 may be activated, in particular after long periods of mechanical ventilation, but to our opinion there is currently no solid evidence that activation of this pathway is responsible for loss of myosin. Our data rather suggest that increased proteolysis through lysosomal autophagy is involved after 8 hours of mechanical ventilation. More importantly, our results show that activation of lysosomal autophagy depends on TLR4 signaling. This is in line with previous data of Doyle et. al. who showed that the TLR4 agonist lipopolysaccharide induces lysosomal autophagy in cultured muscle cells [20].

Of note, after 8 hours of mechanical ventilation, wild-type mice exhibited mild metabolic acidosis, despite insignificant difference in $PaCO_2$ and HCO_3^-. Unfortunately, this could not be prevented as sequential blood gas analysis is not feasible in mice due to low circulating volume. However, it is unlikely that this mild acidosis explains the differences in myosin and cytokine analysis between groups. Previous studies have demonstrated myosin loss after mechanical ventilation in nonacidotic animals [37,39].

Endogenous ligands for TLR4 during mechanical ventilation

Two other questions that remain to be solved are the nature and origin of the ligands that activate TLR4 during mechanical ventilation. Evidence is accumulating that TLR4 can be activated by non-microbial molecules such as endogenous ligands [40] including hyaluronan and heat shock protein 70 [41,42]. Interestingly, these ligands are released from the lung upon mechanical ventilation with high tidal volumes [43,44]. In the current study we choose to ventilate with relatively low tidal volumes, as we wanted to resemble the clinical setting. Nevertheless, a recent study from our lab showed that even low-tidal volume mechanical ventilation results in the appearance of TLR4 ligands in the bronchoalveolar lavage fluid [11]. An attractive hypothesis is therefore that TLR4's in diaphragm are activated by ligands released from the mechanically ventilated lung.

Clinical implications

Respiratory muscle weakness plays an important role in difficult weaning from mechanical ventilation. Recently, the development of respiratory muscle atrophy in mechanically ventilated humans has been demonstrated [1]. Today, no proven strategies are available to prevent or reverse ventilator-induced respiratory muscle atrophy. The current study provides a rationale to test the effects of TLR4 antagonists on ventilator-induced muscle atrophy. Interestingly, a phase 2 trial with the TLR4 antagonist eritoran tetrasodium (Eisai Research Institute of Boston, Andover, MA), showed a trend towards lower mortality rate in severe septic patients [45]. However, this paper did not study the effects of eritoran on duration of mechanical ventilation or respiratory muscle function. Ideally, TLR4 should be selectively blocked in the diaphragm muscle without compromising the innate immune system.

CONCLUSION

In conclusion, controlled mechanical ventilation induces loss of myosin, autophagy and an increased expression of cytokines in the diaphragm muscle. The present study demonstrates that TLR4 signaling is involved in eliciting this response. These findings may prove helpful in the development of strategies to attenuate ventilator-induced diaphragm dysfunction.

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Chapter 5

Hypercapnia attenuates ventilator-induced diaphragm atrophy and modulates dysfunction

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ABSTRACT

Introduction Diaphragm weakness induced by prolonged mechanical ventilation may contribute to difficult weaning from the ventilator. Hypercapnia is an accepted side effect of low tidal volume mechanical ventilation, but the effects of hypercapnia on respiratory muscle function are largely unknown. The present study investigated the effect of hypercapnia on ventilator-induced diaphragm inflammation, atrophy and function.

Methods Male Wistar rats (n=10 per group) were unventilated (CON), mechanically ventilated for 18 hours without (MV) or with hypercapnia (MV+H, $Fico_2$ = 0.05). Diaphragm muscle was excised for structural, biochemical and functional analyses.

Results Myosin concentration in the diaphragm was decreased in MV versus CON, but not in MV+H versus CON. MV reduced diaphragm force by ~22% compared with CON. The force generating capacity of diaphragm fibers from MV+H rats was ~14% lower compared with CON. Inflammatory cytokines were elevated in diaphragm of MV rats, but not in MV+H group. Diaphragm proteasome activity did not significantly differ between MV and CON. However, proteasome activity in the diaphragm of MV+H was significantly lower compared with CON. LC3B-II a marker of lysosomal autophagy was increased in both MV and MV+H. Incubation of MV+H diaphragm muscle fibers with the antioxidant dithiothreitol restored force generation of diaphragm fibers.

Conclusions Hypercapnia partly protects the diaphragm against adverse effects of mechanical ventilation.

INTRODUCTION

It is well-known that controlled mechanical ventilation adversely affects the respiratory muscles [1, 2]. For instance, Levine and colleagues demonstrated that, in brain dead patients, 18 – 69 hours mechanical ventilation is associated with profound atrophy of the diaphragm [1]. Enhanced proteolysis [3] and pro-inflammatory cytokines [4] have been implicated in the development of ventilator-induced diaphragm dysfunction.

Hypercapnia is a well-tolerated side effect of low tidal volume ventilation in patients with acute respiratory distress syndrome. Moreover, experimental studies indicate that hypercapnic acidosis may exert beneficial effects by reducing inflammation and lung injury during mechanical ventilation [5-8]. For instance, in rats with ventilator-induced lung injury, hypercapnic acidosis reduced interleukin (IL)-6 and tumor necrosis factor-alpha in bronchoalveolar lavage fluid compared with normocapnia [6]. Recently, Jung and colleagues demonstrated that in pigs hypercapnic acidosis prevents the loss of diaphragm force induced by controlled mechanical ventilation [9]. The latter study was largely descriptive and the effects of hypercapnic acidosis on downstream mechanisms of ventilator-induced diaphragm dysfunction remain largely unknown [10]**.** Inflammatory and oxidative pathways have been implicated in the development of skeletal muscle atrophy and dysfunction [4, 9-12]. For instance, we have reported that IL-6 may be an important mediator in sepsis-induced skeletal muscle atrophy [11].

Accordingly, the following hypotheses were tested in the present study. First, hypercapnic acidosis attenuates ventilator-induced diaphragm atrophy and dysfunction. Second, hypercapnic acidosis inhibits upregulation of inflammatory cytokines and proteolysis in the diaphragm of mechanically ventilated rats.

To test these hypotheses healthy rats were randomized to an unventilated group, controlled mechanical ventilation during normocapnia or mechanical ventilation under hypercapnic conditions. Diaphragm atrophy, function, inflammation and proteolysis were assessed. Based on these results, additional experiments were performed to assess the role of oxidative protein modification on contractile function of the diaphragm.

MATERIALS & METHODS

Design

Animal experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care.

Experiments were performed in male Wistar rats (Harlan, Horst, The Netherlands) randomly divided in three groups (N = 10 per group): control group (CON, bodyweight 313 ± 20 gram), mechanical ventilation group (MV, bodyweight 294 ± 15 gram) and mechanical ventilation with hypercapnia group (MV+H, bodyweight 313 ± 12 gram). The CON group was anesthetized with pentobarbital (50 mg/kg) and sacrificed without being mechanically ventilated.

Rats allocated to mechanical ventilation were anesthetized with an intra-peritoneal injection of pentobarbital (induction 50 mg/kg), orally intubated and ventilated (ventilator UB 7025 from Hugo Sachs, March-Hugstetten, Germany) as described previously [13]. Briefly, in all mechanically ventilated animals a catheter was inserted under sterile conditions in the carotid artery for continuous measurement of blood pressure and periodic blood sampling (at ½, 2, 4, 8, 12, 16 and 18 hours; i-STAT, Blood Gas Analyzer, Abbott, Hoofddorp, The Netherlands). Tidal volume was set at 6 ml/kg bodyweight, respiratory rate of 110/min, positive end-expiratory pressure of 1.5 cm H_2O and inspired oxygen fraction of 0.45. Hypercapnic acidosis was induced by adding $Fico_2$ 0.05 in the MV+H group. Ventilated rats were fed iso-caloric AIN-76 rodent diet (SSNIFF, Soest, The Netherlands) through an oro-gastric tube and received a continuous intra-venous doses pentobarbital (10 mg/kg/hour) via a tail vein catheter as previously described [14]. To compensate for loss of circulating volume, Ringer's solution at 2 ml/hour was administered intravenously. Body temperature was kept between 36.0°C and 37.0°C using a heating pad [13]. All animals survived the 18 hours of mechanical ventilation without complications.

Tissue collection

Immediately after anesthesia (CON) or after 18 hours of mechanical ventilation (MV and MV+H groups), rats were exsanguinated and a combined thoracotomy and laparotomy was performed, as described previously [13]. Left and right hemidiaphragm tissue was rinsed with the left part quickly frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis and the right hemidiaphragm submersed in cooled Krebs solution at pH 7.4 for single fiber isolation, as described previously [15].

Myosin heavy chain isoform and concentration

Content of myosin heavy chain was analysed in diaphragm muscle homogenates using standard Western blotting as described previously [4, 19]. Myosin heavy chain content on blot was corrected for loaded amount of muscle weight. For a more accurate determination of myosin heavy chain content in diaphragm, myosin heavy chain concentration (i.e. amount of myosin per muscle volume) was measured in diaphragm single fibers. Determination of myosin heavy chain isoform composition and concentration in same diaphragm fibers as used for contractile measurements by means of SDS-PAGE, was described previously [16] and adapted from Geiger et al. [17]. Briefly, single fibers were placed in a sample buffer, thereafter run on gel and afterwards silver stained and quantified. Since only 5 diaphragm fibers in each group expressed the slow isoform of myosin heavy chain, these were excluded from further analysis. Accordingly, all fibers were classified as fast (2x and 2b) typed fibers.

Skinned fiber: cross sectional area, contractile measurements

Cross sectional area (CSA) and maximal active force generation of skinned single fibers isolated from the diaphragm muscle were determined (5 - 6 fibers per rat) as described previously [16].

To obtain single fibers, a rectangular bundle from the central costal region of the right hemidiaphragm was dissected, parallel to the long axis of the muscle fibers. The muscle bundle was chemically skinned, i.e. permeabilization of lipid membranes. Subsequently, single fibers were isolated from the muscle bundle, attached to aluminium foil clips, and mounted in a flow-through acrylic chamber on two hooks connected to a force transducer (model AE-801; SensoNor, Horten, Norway) and a servomotor (model 308B, Aurora Scientific, Aurora, ON, Canada). Sarcomere length was set at 2.4 μm as the optimal length for force generation. Muscle fiber CSA area was deduced from fiber width and depth measurements using a reticule in the microscope eyepiece. Maximum isometric force was determined by measuring force after perfusing the experimental chamber with, successively, pCa 9.0 and pCa 4.5 solutions. Maximum specific force was derived from dividing maximum isometric force by fiber CSA [18].

Cytokines

Concentrations of IL-1beta, IL-6, keratinocyte-derived chemokine, IL-10 and tumor necrosis factor-alpha in diaphragm homogenates were analysed with ELISA as described previously (R&D Systems, Minneapolis, Minnesota) [4].

E3-ligases, Ubiquitin-proteasome pathway, Autophagy and Transcription

To assess involvement of proteolysis we measured 20S proteasome proteolytic activity as described previously [19]. Levels of muscle specific E3-ligases, markers for muscle atrophy, MAFbx and MuRF-1 mRNA were determined using qPCR as previously described [19]. Concentration of ubiquitinated myosin and total myosin were determined as described before in whole diaphragm muscle homogenates, using standard Western blotting [4, 19]. To study the role of lysosomal autophagy, the content of autophagy markers Light Chain 3B-II (LC3B-II) and beclin-1 were measured by standard Western blotting as described previously [4], using specific antibodies against LC3B (anti-LC3B-II 2775, Cell signaling Technology, Danvers, MA) and beclin-1 (anti-beclin-1, PRS3613, Sigma-Aldrich, Saint Louis, MO). Gels were equally loaded with 40 µg protein per sample. Optical density of LC3B-II and beclin-1 bands on blot were quantified using Odyssey scan and Odyssey application software version 2.1 (LI-COR, Lincoln, NE). In addition, myosin heavy chain (MyHC) expression of isoforms I, IIa, IIb and IIx was analysed according to methods described for MAFbx and MuRF-1 mRNA. Forward and reverse oligonucleotides used were as follows: MyHC I: 5'–GCCAAGAGCCGTGACATTGGC–3' and 5'–CTGCCTGAAGGTGCTGTTTCA–3', MyHC IIa 5'–TATCCTCAGGCTTCAAGATTTG–3' and 5'–TAAATAGAATCACATGGGGACA–3', MyHC IIB 5'CACACCAAAGTCATAAG CGAA–3' and 5'–CCTTGATATACAGGACAGTGA–3', MyHC IIX 5'–TGATCGATCCAAAGCAGG–3' and 5'–CTCCCAAAGTCGTAAGTA–3'.

Effect of oxidation/reduction state on force generation and the presence of oxidative stress

To investigate involvement of reversible protein modifications by oxidative agents, additional experiments were performed using reducing agent Dithiothreitol (DTT) (Sigma, Zwijndrecht, the Netherlands). Maximal active force generation of skinned diaphragm fibers (n=16) was determined before and after 20 min incubation with DTT (10mM in relax solution) [20].

To investigate the presence of oxidative stress, HNE (4-Hydroxy-2-nonenal) was measured using a specific antibody against HNE (anti-HNE 393206, Calbiochem, Darmstadt, Germany). Diaphragm muscle homogenates were prepared as described previously [4]. Optical density of HNE bands on blot were quantified using Odyssey scan and Odyssey application software version 2.1 (LI-COR, Lincoln, NE).

Data treatment and statistical methods

Differences among groups were analysed with one-way ANOVA and Student-Newman-Keuls post hoc testing if appropriate. A two-sided paired Student t-test was performed to evaluate the statistical difference between maximal forces before and after DTT incubation. Difference among groups regarding time courses of pH, $Paco_2$, Pao_2 and mean arterial pressure (MAP) were performed with a two-way ANOVA. GraphPad Prism was used to conduct statistical analysis (GraphPad Software Inc., San Diego, CA). A probability level of $p < 0.05$ was considered statistically significant. All data are presented as mean ± SE, except bodyweight that is presented as mean ± SD.

RESULTS

Animal characteristics

Animals allocated to ventilated groups were hemodynamically stable during the 18 hours of ventilation. No respiratory efforts were observed clinically or from the pressure tracing of the ventilator. Figure 1A-B-C-D shows the course of pH, $Paco_2$, Pao_2 and MAP during mechanical ventilation. As expected MV+H rats developed respiratory acidosis that was partly metabolically compensated. Plasma HCO_3^- after 18 hours of mechanical ventilation was 33.6 ± 0.7 mmol/L in MV and 43.7 ± 3.1 mmol/L in MV+H.

Hypercapnia and diaphragm structure and function

Hypercapnia prevented the loss of myosin in ventilated rats (Figure 2A). Subsequently, for a more precise measurement of muscle atrophy, myosin heavy chain concentration was measured in diaphragm single fibers. Accordingly, mechanical ventilation significantly decreased myosin concentration in diaphragm muscle fibers (Figure 2B). Hypercapnia prevented the loss of myosin induced by mechanical ventilation, in fact, mean myosin concentration was higher in diaphragm fibers from MV+H group compared with CON (Figure 2B).

Besides myosin concentration, muscle fiber CSA was measured as an additional marker for atrophy. Diaphragm fiber CSA was reduced by ~25% in MV animals (Figure 2C, $P < 0.05$ versus CON). The reduction of CSA was less pronounced, i.e. ~15%, in diaphragm fibers from MV+H rats ($P = 0.06$, versus CON).

Mechanical ventilation reduced specific force generation of diaphragm muscle fibers by ~22% compared with CON (Figure 2D, $P < 0.05$). The force generating capacity of diaphragm fibers from MV+H group was ~14% lower compared with CON, but this difference did not reach statistical significance ($P = 0.16$).

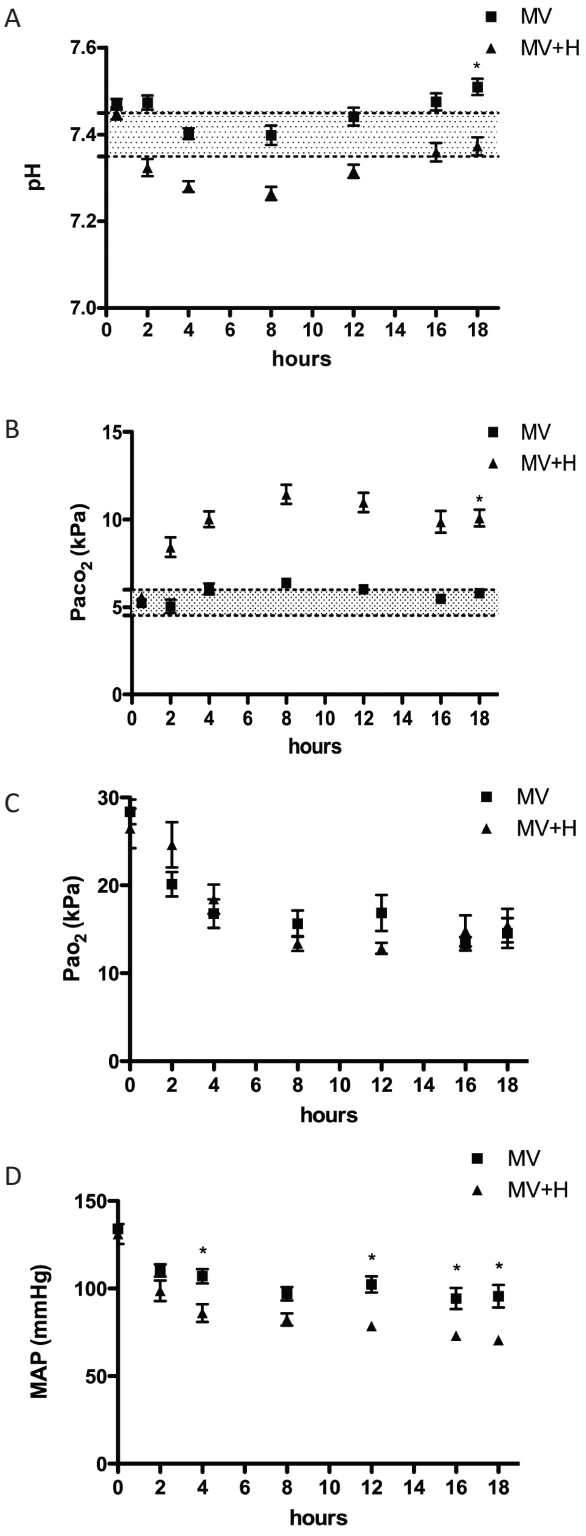

Figure 1 A) Course of blood pH during 18 hours of mechanical ventilation. As expected increasing $Fico_2$ reduces blood pH. * Two-way repeated measure analysis showed that pH values over time were significantly different between groups.

B) Course of blood CO_2 during 18 hours of mechanical ventilation. CO_2 was significantly increased at the end of experiment in hypercapnic rats.* Two-way repeated measure analysis showed that CO_2 values over time were significantly different between groups.

C) Course of blood O_2 during 18 hours of mechanical ventilation. O_2 was not significantly different between mechanically ventilated groups.

D) Course of blood MAP during 18 hours of mechanical ventilation. MAP was significantly lower at 4 hours and at the end of experiment in hypercapnic rats. * Two-way repeated measure analysis showed that MAP was significantly different between groups at 4, 12, 16 and 18 hours of mechanical ventilation.

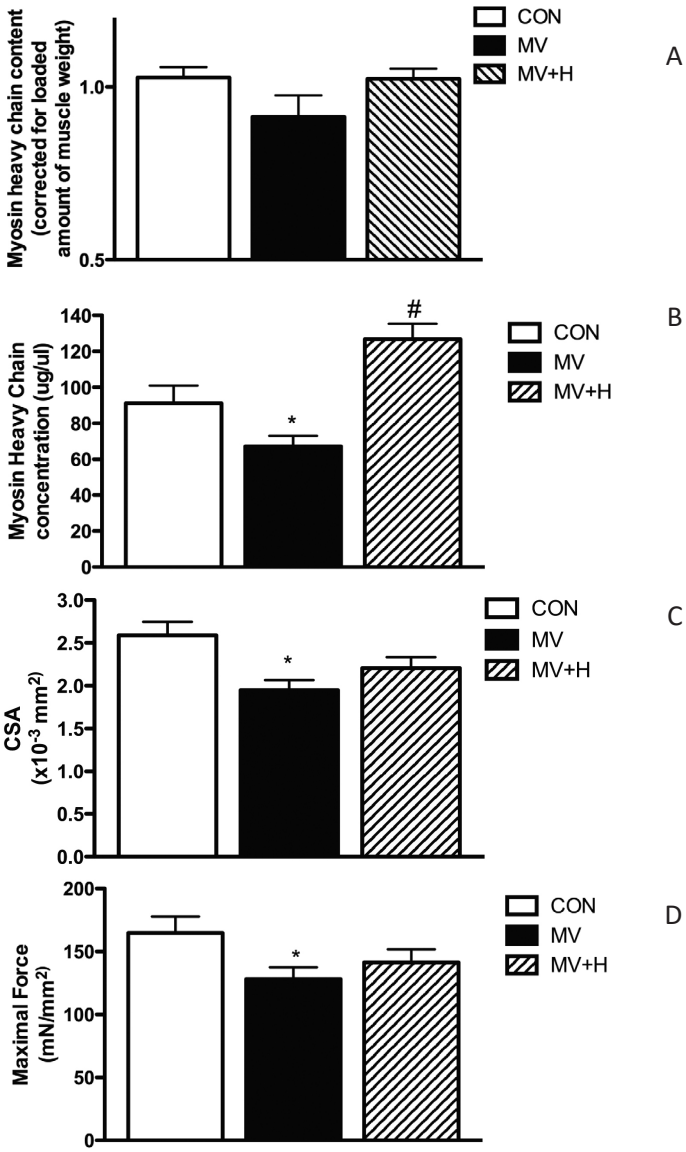

Figure 2 A) Myosin heavy chain content per muscle weight in diaphragm of MV showed a decreased trend compared with CON and MV+H, but was not significantly different between groups. B) Myosin heavy chain concentration in diaphragm single fibers. Control diaphragm fibers (n = 51) showed a higher concentration of myosin heavy chain compared with fibers from ventilated rats (n = 57) (* vs. control; $P < 0.05$). Fibers from hypercapnic rats (n = 53) showed higher concentration of myosin heavy chain versus fibers from normocapnic ventilated animals (# vs. MV and vs. control; $P < 0.05$). C) Diaphragm fast typed fibers cross sectional area. Cross sectional area was reduced in MV animals versus CON (* $P < 0.05$). The reduction of cross sectional area was less pronounced in diaphragm fibers from MV+H rats. D) Maximal force corrected for cross sectional area. Force of fibers from 18 hours mechanical ventilated rats were significantly lower compared with control (* $P < 0.05$).

Inflammation

Mechanical ventilation resulted in increased levels of inflammatory cytokines in the diaphragm. For instance, IL-1beta, IL-6 and tumor necrosis factor-alpha increased by 37%, 109%, and 35%, respectively compared with CON. This inflammatory response was largely abolished in MV+H animals (Figure 3).

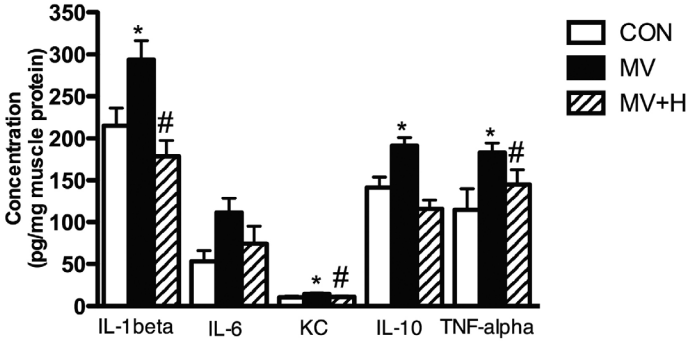

Figure 3 Cytokine levels in diaphragm homogenates. Mechanical ventilation significantly increased levels of IL-1beta, KC, IL-10 and tumor necrosis factor-alpha (* vs. control; $P < 0.05$). Hypercapnia largely abolished ventilator-induced inflammation in the diaphragm after 18 hours of MV (# vs. MV $P < 0.05$ for IL-1beta, KC and TNF-alpha). MV+H versus CON was not significantly different for all cytokines

E3-ligases, Ubiquitin-proteasome pathway, Autophagy and Transcription

To test whether the protective effects of hypercapnia on diaphragm myosin concentration in the ventilation group were mediated by inhibition of proteolytic pathways, we subsequently analysed essential segments of the ubiquitin-proteasome pathway and a key lysosomal autophagy marker. Mechanical ventilation significantly enhanced mRNA expression of the E3-ligases MuRF-1 (11,4 fold expression in MV versus CON, $P < 0.05$) and MAFbx (11.9 fold expression in MV versus CON, $P < 0.05$) in the diaphragm. Hypercapnia did not affect ventilator-induced activation of these E3-ligases in the diaphragm (13.1 fold increase in MuRF-1 and 9.1 fold increase in MAFbx versus CON, both $P < 0.05$). A trend for an increased amount of ubiquitinated myosin molecules was found in MV (Figure 4A/B, 128% in MV versus CON) and MV+H group (138% in MV+H versus CON) compared with control, but these differences did not reach statistical significance. Diaphragm proteasome activity was not significantly different between MV and CON (Figure 4C). However, proteasome activity in the diaphragm of the hypercapnic ventilated group was 24% lower compared with CON $p < 0.05$ (Figure 4C). Light Chain 3B-II, a marker for autophagy, was increased in both ventilated groups compared with CON (Figure 4D/E). Another marker for autophagy, beclin-1, was not different between groups. Myosin expression in relation to the expression of tubulin was not different between groups for myosin heavy chain isoforms I, IIa, IIb and IIx (Figure 5).

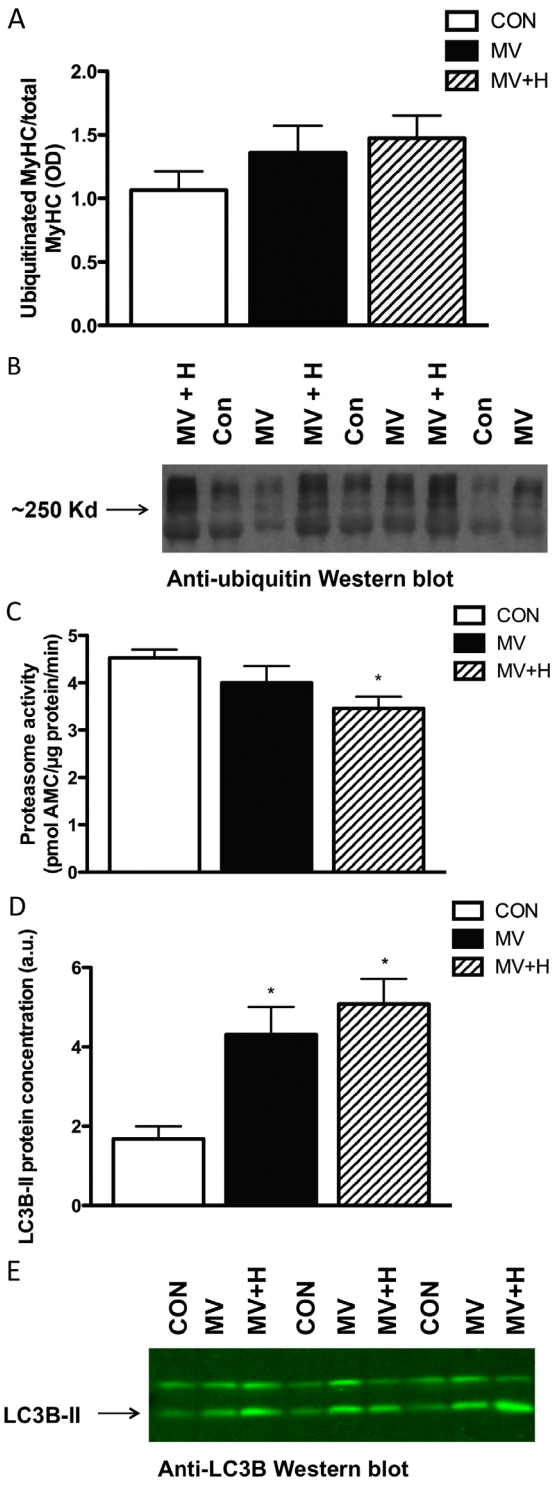

Figure 4 A) Concentration of ubiquitinated myosin per total myosin in diaphragm of MV and MV+H showed an increased trend compared with control, but was not significantly different between groups.
B) Representative Western blot of ubiquitinated myosin.
C) 20S Proteasome activity in diaphragm. Hypercapnic ventilated animals demonstrate decreased activity versus control animals (* $P < 0.05$).
D) LC3B-II content. Both ventilated and hypercapnic ventilated animals were significantly different compared with control (* $P < 0.05$).
E) Representative Western blot against LC3B-II.

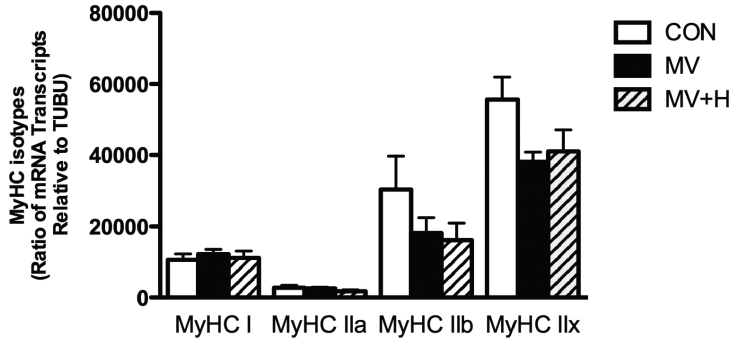

Figure 5 Expression of mRNA Myosin Heavy Chain isoforms I, IIa, IIb and IIx per tubulin in rat diaphragm. Transcription was not different between groups.

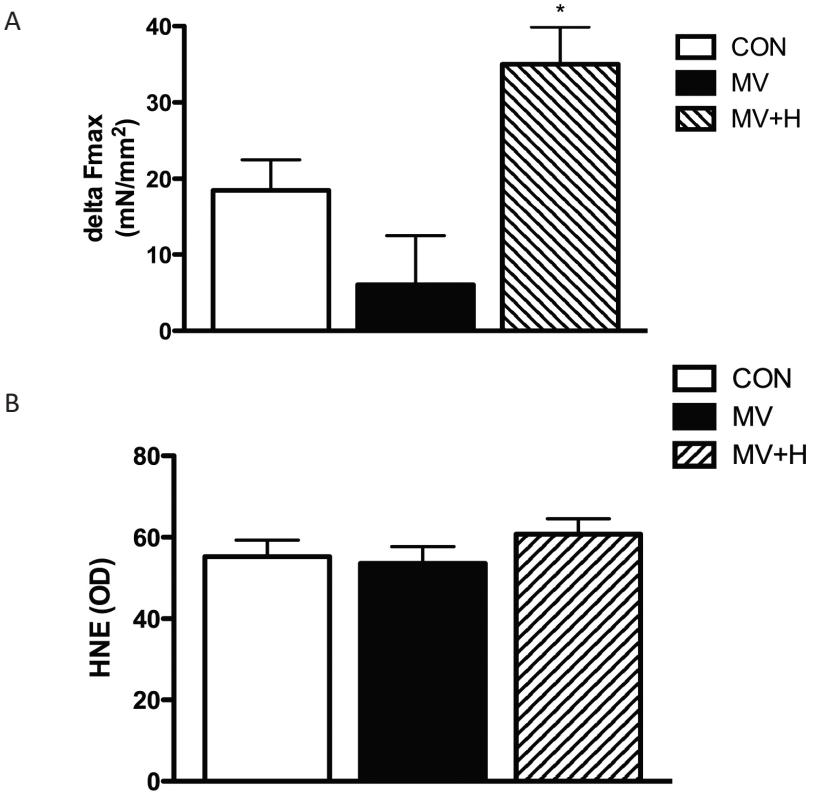

Figure 6 A) Delta force, corrected for cross sectional area, in single fibers before and after incubation with DTT. Delta force in MV+H (both n = 16) versus CON and MV was significant increased (* $P < 0.05$). B) 4-Hydroxy-2- nonenal (HNE) optical density (OD) in diaphragm was not significantly different between groups.

Effect of oxidation/reduction state on force generation

An unexpected finding was that the protective effect of hypercapnia on contractile protein concentration was only partly accompanied by a protective effect on force generation (see Figure 2B and 2D respectively).

Reduced force generation without the loss of contractile protein in hypercapnic rats suggests posttranslational modifications of the contractile proteins. Oxidative modifications are known to affect respiratory muscle force and indeed mechanical ventilation has been associated with oxidative stress [12, 21]. Accordingly, we investigated if reversible protein oxidation is involved in the reduced force generating capacity of diaphragm fibers in the MV+H group. Incubation of diaphragm muscle fibers from hypercapnic ventilated rats with the antioxidant DTT increased force generation by 27% ($P < 0.05$ versus CON, Figure 6A). In contrast, DTT did not affect force generation of diaphragm fibers from MV group (Figure 6A). Concentration of HNE, a marker for reversible oxidative stress, in diaphragm was not different between groups (Figure 6B).

DISCUSSION

Previous studies have shown that controlled mechanical ventilation has detrimental effects on diaphragm structure and function. In the present study we report beneficial effects of hypercapnic acidosis on the diaphragm during controlled mechanical ventilation. The most important new findings are that 1) Mechanical ventilation under hypercapnic conditions prevents loss of myosin in the diaphragm. 2) Hypercapnic acidosis largely abolishes ventilator-induced inflammation in the diaphragm. 3) Hypercapnic acidosis does not attenuate the immediate effects of mechanical ventilation on diaphragm single fiber function. 4) The antioxidant DTT restores diaphragm muscle function in the hypercapnic ventilated group.

Effects of hypercapnia on ventilator-induced diaphragm dysfunction

In line with previous studies in animals and humans the present findings confirm that diaphragm weakness upon prolonged mechanical ventilation is accompanied by increased levels of inflammatory mediators in the diaphragm, activation of proteolytic pathways and loss of contractile proteins [1, 3, 4, 22-26].

Hypercapnic acidosis has been shown to impair diaphragm function [27-30]. Very recently however, Jung et al. showed that hypercapnic acidosis protects against diaphragm dysfunction in piglets after 72 hours of mechanical ventilation [9]. In apparent contrast, we observed a decreased force in single fibers after 18 hours of hypercapnic mechanical ventilation. Of note, Jung et al. used an in vivo model of

diaphragm function, by stimulating phrenic nerves while the present study looked specifically at contractile proteins and their function. A plausible explanation for this discrepancy is that recovery of diaphragm function upon hypercapnia becomes significant after 18 hours of mechanical ventilation. Interestingly, in the study by Jung et al. diaphragm function was also decreased after 12 hours in both normal and hypercapnic ventilated groups [9]. Although that study clearly showed a potential beneficial effect of hypercapnia after 72 hours of mechanical ventilation, the mechanisms by which hypercapnia exerts these effects were not studied [10]. An important finding of the present study is that hypercapnia completely prevented mechanical ventilation induced loss of myosin (Figure 2B). Preservation of myosin most likely resulted from reduced degradation, as mechanical ventilation under hypercapnic conditions was associated with reduced 20S proteasomal activity and a trend of increased ratio of ubiquitinated myosin over total myosin (Figure 4A/B). Of note, the correction for total myosin presumably underestimates the actual amount of ubiquitinated myosin in MV+H, since the concentration of myosin is increased in this group. We observed a trend of decreased myosin content per muscle weight in ventilated rats, but not in hypercapnic ventilated rats. Since myosin is one of the major proteins in skeletal muscle, correcting myosin content for total muscle weight is expected to underestimate the loss of myosin. Therefore we chose to analyse myosin loss more precisely by measuring myosin concentration (i.e. the amount of myosin per muscle volume) in single fibers. Remarkably, concentration of myosin in diaphragm single fibers of hypercapnic ventilated animals was even higher than in controls. This can be explained by the fact that the CSA was slightly decreased in the hypercapnic ventilated group compared with control (Figure 2C). Accordingly, myosin concentration increases when myosin content remains stable, but the volume of the muscle fiber, reflected by CSA, decreases. These data suggest that loss of myosin and loss of muscle fiber circumference are likely not tightly linked [31]. Preservation of myosin in the hypercapnic ventilated group however did not protect the diaphragm muscle from reduced force generation, implicating more dysfunctional myosin in hypercapnic ventilated rats. This discrepancy is most likely the result of posttranslational modifications of contractile proteins. Indeed, the antioxidant DTT increases force-generating capacity of diaphragm fibers from the MV+H group, indicating that oxidative protein modifications play a role in loss of force induced by controlled mechanical ventilation under hypercapnic conditions. Notably, DTT did not improve force generation in muscle fibers in the group ventilated under normocapnic conditions (Figure 6A). There are two reasonable explanations for the different effect of DTT on normocapnic and hypercapnic fibers. First, as hypercapnia reduced proteasomal activity, degradation of post-translationally modificated (i.e. oxidated) myosin in the

diaphragm is attenuated. In line with that, hypercapnic ventilation tended to increase levels of ubiquitinated myosin. In both ventilated groups, an increase of LC3B-II protein suggests activation of autophagy. However the autophagy marker beclin-1 was not increased. Such a discrepancy has also been observed recently in the diaphragm in septic mice [32], suggesting that autophagy can occur independent of beclin-1.

Secondly, hypercapnia may be associated with oxidative stress. Indeed, Arbogast et al. demonstrated that elevated CO_2 promotes oxidant activity in diaphragm muscle bundles [33]. We attempted to detect oxidative modifications of muscle proteins by measuring level of HNE. However, we did not see any difference between groups in HNE levels, which does not exclude that other oxidative modifications, e.g. formation of disulfide bonds, have occurred.

In the present study, hypercapnic ventilated animals showed an acidemia during most time of mechanical ventilation period (Figure 1A). The question that remains is if the effects on the diaphragm observed in the present study are the result of hypercapnia or the combination of hypercapnia and acidemia. We have recently concluded that most beneficial effects appear to be pH mediated in both in vivo and in vitro experiments, although synergistic effects of acidemia and hypercapnia have also been observed [8]. Besides its direct effects on muscle function, hypercapnia has systemic effects. For example, by a rightward shift of the oxyhemoglobin dissociation curve, oxygen release to the tissues is facilitated [8] and by sympathetic stimulation increasing cardiac output and MAP [8, 9]. Unexpectedly, we observed a significantly decreased MAP in hypercapnic ventilated animals. Whether systemic effects of hypercapnia could affect diaphragm function during controlled mechanical ventilation is however uncertain.

Effect of cytokines in muscle and hypercapnia

Recent work from our lab showed that 8 hours of mechanical ventilation induced an inflammatory response in the mouse diaphragm [4]. The current study confirms this observation in the mechanically ventilated group. Hypercapnia largely abolished the increases levels of inflammatory cytokines in the diaphragm of ventilated rats (Figure 3). Attenuating the inflammatory response by hypercapnia might protect the diaphragm from atrophy during mechanical ventilation. Previous studies have shown that hypercapnia dampens the inflammatory response, probably through inhibition of NF-κB, in acute lung injury [6, 8, 34]. The remaining question is whether myosin preservation and depression of proteasome activity in the diaphragm of the hypercapnic ventilated group is mediated by attenuation of the inflammatory response or by a direct effect of hypercapnia on NF-κB inhibition. Both tumor necrosis factor-alpha and IL-1beta have been associated

with skeletal muscle atrophy [35-37]. Tumor necrosis factor-alpha induces protein loss in cultured C2C12 skeletal muscle cells [36] and exposure of skeletal muscle cells to IL-1beta for 48 hours induces muscle atrophy [35]. Of note, although IL-10 is an anti-inflammatory cytokine, its production is induced by pro-inflammatory mediators and therefore changes in IL-10 levels, as observed in the current study, can also be considered as a surrogate marker of the magnitude of the pro-inflammatory response [38, 39].

Clinical relevance

Hypercapnic acidosis has been shown to attenuate ventilator-induced lung injury and inflammation [8]. The present study shows that therapeutic hypercapnia may also protect the diaphragm from adverse events associated with controlled mechanical ventilation. This is of potential clinical importance, since respiratory muscle dysfunction frequently occurs in mechanically ventilated patients and is associated with prolonged weaning, increased morbidity and mortality in ICU patients [40-42]. Besides training, no specific treatment is currently available for muscle weakness in the critically ill. Therefore, therapeutic hypercapnia during mechanical ventilation in order to prevent ventilator-induced diaphragm weakness deserves further study.

Our study also generates new hypotheses on the role of antioxidants in the prevention of ventilator-induced respiratory muscle dysfunction. Betters et al. have shown that administration of antioxidants at time of initiation of mechanical ventilation is effective in the prevention of diaphragm dysfunction [12]. Our data indicate that once ventilator-induced diaphragm dysfunction has occurred antioxidants might not be effective (Figure 6A), unless mechanical ventilation is performed under hypercapnic conditions. This study indicates that the timing of antioxidant administration may be of critical importance as well as the associated conditions (i.e. the presence of hypercapnia).

CONCLUSION

The present study shows that mechanical ventilation under hypercapnic conditions protects the diaphragm from ventilator-induced diaphragm-atrophy and enables antioxidants to restore diaphragm muscle force after 18 hours of mechanical ventilation.

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Chapter 6

Levosimendan affects oxidative and inflammatory pathways in the diaphragm of ventilated endotoxemic mice

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ABSTRACT

Introduction Controlled mechanical ventilation and endotoxemia are associated with diaphragm muscle atrophy and dysfunction. Oxidative stress and activation of inflammatory pathways are involved in the pathogenesis of diaphragm dysfunction. Levosimendan, a cardiac inotrope, has been reported to possess anti-oxidative and anti-inflammatory properties. The current study aimed to investigate the effects of levosimendan on markers for diaphragm nitrosative and oxidative stress, inflammation and proteolysis in a mouse model of endotoxemia and mechanical ventilation.

Methods Three groups were studied: 1. unventilated mice (CON, n=8), 2. mechanically ventilated endotoxemic mice (MV LPS, n=17) and 3. mechanically ventilated endotoxemic mice treated with levosimendan (MV LPS+L, n=17). Immediately after anesthesia (CON) or after 8 hours of mechanical ventilation, blood and diaphragm muscle were harvested for biochemical analysis.

Main results Mechanical ventilation and endotoxemia increased expression of inducible nitric oxide synthase (iNOS) mRNA and cytokine levels of interleukin (IL)-1beta, IL-6 and keratinocyte-derived chemokine (KC), and decreased IL-10 in the diaphragm, but had no effect on protein nitrosylation and 4-hydroxy-2-nonenal-protein concentrations. Levosimendan decreased nitrosylated proteins by 10% ($p < 0.05$) and 4-hydroxy-2-nonenal-protein concentrations by 13**%** ($p < 0.05$), but augmented the rise of iNOS mRNA by 47% ($p < 0.05$). Levosimendan did not affect the inflammatory response in the diaphragm induced by mechanical ventilation and endotoxemia.

Conclusions Mechanical ventilation in combination with endotoxemia results in systemic and diaphragmatic inflammation. Levosimendan partly decreased markers of nitrosative and oxidative stress, but did not affect the inflammatory response.

INTRODUCTION

Respiratory muscle dysfunction frequently develops in critically ill patients [1-4]. Clinical entities associated with respiratory muscle dysfunction in these patients include systemic inflammation [3, 5] and controlled mechanical ventilation [6]. Recent data obtained from both animal and human studies have provided insight in the biochemical pathways underlying muscle atrophy and dysfunction in these patients [1, 7-9]. Nitrosative and oxidative protein modifications are increased in the diaphragm during both mechanical ventilation and experimental sepsis [10, 11]. Moreover, protein degradation is increased in the diaphragm of mechanically ventilated animals and patients [2, 12, 13]. Inflammatory cytokines are well-known modulators of muscle protein turnover during endotoxemia [14, 15] and are increased in diaphragm after mechanical ventilation [16, 17]. For example, tumor necrosis factor (TNF)-alpha activates the proteolytic enzymes caspase 8 and 3 in diaphragm of mice exposed to lipopolysaccharide (LPS) [5]. Accordingly, strategies that reduce oxidative stress or inflammation may limit respiratory muscle dysfunction due to endotoxemia and mechanical ventilation [11, 17].

Levosimendan is a cardiac inotrope approved for the treatment of heart failure in many countries world-wide. We have recently shown that levosimendan also improves respiratory muscle function in healthy subjects and patients with chronic obstructive pulmonary disease [18, 19]. The mechanisms of levosimendan include calcium sensitization of the contractile proteins and vasodilation through activation of ATP sensitive K-channels [20]. Interestingly, previous studies have shown that levosimendan reduces inflammation and also oxidative- and nitrosative stress [21-23]. For instance, in experimental septic rodents, levosimendan decreases IL-1beta [24]. In addition, levosimendan decreases LPS-induced upregulation of IL-6, nitrite production and iNOS protein expression in macrophages and fibroblasts [21]. However, the effects of levosimendan on inflammation and oxidative / nitrosative stress in muscle have not been investigated, despite the importance of these pathways in ICU acquired muscle weakness.

Accordingly, the hypothesis of the current study is that levosimendan dampens both oxidative and nitrosative stress and the inflammatory response in the diaphragm induced by endotoxemia.

MATERIALS & METHODS

Animals

Experiments were performed using male C57BL/6 mice aged 19 ± 0.5 weeks, bodyweight 24 ± 0.3 gram (Charles River, Sulzfeld, Germany).

To test the hypothesis that levosimendan dampens both oxidative and nitrosative stress and the inflammatory response in the diaphragm induced by mechanical ventilation and experimental sepsis, forty-two mice were divided into three groups: 1. Unventilated controls (CON, n=8), 2. mechanical ventilation and LPS (MV LPS, n=17) and 3.) Mechanical ventilation and LPS + levosimendan (MV LPS+L, n=17). The effects of mechanical ventilation on inflammatory markers in mice have been investigated before in our lab [16]. Because patients admitted to the intensive care with endotoxemia often require mechanical ventilation, we used an animal model in which endotoxemia was administered prior to mechanical ventilation. In both groups, LPS (10 microgram) was administered by intra-peritoneal injection directly before initiation of mechanical ventilation.

The final choice for the dose of LPS was based on pilot experiments. In a pilot study we found that administration of 100 ug of LPS resulted in high mortality. Nevertheless, immediately after intraperitoneal injection of LPS bolus, one mouse in MV LPS+L died and was excluded from the study. At the start of mechanical ventilation, mice in the levosimendan group received a dose of levosimendan (Orion Pharma, Espoo, Finland) (24 µg/kg bodyweight in 5% glucose solution) via the tail vein. Subsequently, levosimendan (0,2 µg/kg/min i.v.) was administered during mechanical ventilation [20]. Experiments were approved by the Regional Animal Ethics Committee (Nijmegen, The Netherlands) and performed under the guidelines of the Dutch Council for Animal Care.

Controlled mechanical ventilation

Mice selected for mechanical ventilation were anesthetized and mechanically ventilated as described previously [16]. Briefly, mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine, and atropine (KMA): 7.5 ml per gram of body weight of induction KMA mix (consisting of 1.26 ml ketamine, 100 mg/ml; 0.2 ml medetomidine, 1 mg/ml; 1 ml atropine, 0.5 mg/ml; and 5 ml NaCl, 0.9%). Tidal volume was 8 ml/kg bodyweight, respiratory rate of 170/min, positive end-expiratory pressure of 1.5 cmH_2O and inspired oxygen fraction of 0.45. A sterile catheter was inserted in the carotid artery for continuous blood pressure measurement and blood sampling at the end of the experiment (Blood Gas Analyzer i-STAT, Abbott, Hoofddorp, The Netherlands). To maintain anesthesia, 5.0µl/gram bodyweight of bolus of KMA mix (consisting of 0.72 ml ketamine, 100 mg/ml; 0.08 ml medetomidine, 1 mg/ml; 0.3 ml atropine,

0.5 mg/ml; and 18.9 ml NaCl, 0.9%) was administered through an intraperitoneal catheter, every 30 min. The CON mice were anesthetized and sacrificed without being mechanically ventilated as described previously [16].

Tissue collection

Immediately after anesthesia (CON) or after 8 hours of mechanical ventilation (MV LPS and MV LPS+L), mice were exsanguinated and a combined thoracotomy and laparotomy was performed. Left and right hemidiaphragm tissue were rinsed, quickly frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis [16].

Nitrotyrosine

Protein nitrosylation was evaluated by detection of nitrotyrosine residues by performing SDS–polyacrylamide gel electrophoresis, as described previously [25]. In short, diaphragm muscle was homogenized in ice-cold buffer, (pH 7.2 10 mM Tris/Maleate, 3 mM EGTA, 275 mM sucrose, 0,1 mM dithiothreitol, 2 mg/ml Leupeptine, 2 mg/ml Aprotinine, 10 mg/L Pepstatine A, 0,57 mM phenylmethyl-sulphonylfluoride) using a polytron, followed by 3 cycles of freeze/thawing and 30 minutes centrifugation at 17.000g at 4˚C. Supernatant protein content was measured by Bradford analysis and 20 microgram of proteins in Laemmli buffer were analysed by standard Western blotting protocols. Blots were stained using an anti-nitrotyrosine antibody (clone 1A6; Upstate Biotechnology, Lake Placid, NY) and goat anti-mouse IRDye 800CW (LI-COR, Lincoln, NE). Analysis was done using ODYSSEY scanner and ODYSSEY 2.1 software (LI-COR).

iNOS expression

Messenger ribonucleic acid levels of iNOS mouse: Mm01309902 m1, Applied Biosystems, Carlsbad, CA, USA) were determined by quantitative polymerase chain reaction as described previously [26].

HNE-protein

4-hydroxy-2-nonenal-protein (HNE)-protein was analysed as follows, sample was homogenized in 100 volumes buffer (20 mM Tris pH 7,4, 20 mM EGTA, 1 mM DTT, 0,5 % SDS, 1ul/20 mg Protease inhibitor cocktail (P8340, Sigma, Zwijndrecht, The Netherlands)), by polytron tissue homogenization. Samples were diluted in standard Laemmli sample buffer and 2 minutes boiled. 10 microliter of each sample was analysed by standard Western Blotting protocols. Used antibodies were: anti-HNE (Fluorophore Rabbit pAb, Cat. No. 393206, Calbiochem, Darmstadt, Germany); anti-Actin (A2066, Sigma-Aldrich, St. Louis, MO, USA); second antibody IRDye®800CW Goat anti-Rabbit IgG (926-32211, LI-COR Biosciences, Lincoln,

Nebraska, USA). Odyssey scan and Odyssey application software version 2.1 (LI-COR, Lincoln, NE) were used for analysis of HNE-protein signal.

Cytokines in diaphragm and plasma

Levels of IL-1beta, IL-10, TNF-alpha, IL-6 and KC in the diaphragm and IL-1beta, IL-10, TNF-alpha, IL-6 and KC in plasma were analysed by enzyme-linked-immunosorbent assay as published previously [27]. To determine cytokine levels in the diaphragm, the muscle was homogenized in 100 volumes of ice-cold buffer, pH 7.2 (10 mM Tris/Maleate, 3 mM EGTA, 275 mM sucrose, 0,1 mM dithiothreitol, 2 mg/ml Leupeptine, 2 mg/ml Aprotinine, 10 mg/L Pepstatine A, 0,57 mM phenylmethylsulphonylfluoride) three cycles of freezing and thawing and centrifuged at 17.000 G at 4°C for 30 minutes [16]. Lower detection limits were IL-10, 40 pg/ml for IL-1beta; 32 pg/ml for TNF-alpha; 160 pg/ml for IL-6 and for KC.

Caspase-3 activity

To assess involvement of proteolysis we measured caspase-3 activity as described previously [26]. The caspase-3 activity was determined by measuring the generation of the fluorogenic cleavage product methylcoumarylamide from the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by spectrophotometry.

MuRF-1 and MAFbx expression

Messenger ribonucleic acid levels of muscle RING-finger (MuRF-1) and Muscle Atrophy Factor box (MAFbx) were determined by quantitative polymerase chain reaction [26]. Levels of MuRF-1 and MAFbx messenger ribonucleic acid were normalized to glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid. Forward and reverse oligonucleotides were used as following: MuRF-1, 5'-CAACCTGTGCCGCAAGTG-3' and 5'-CAACCTCGTGCCTACAAGATG-3', MAFbx, 5'-GACTGGACTTCTCGACTGCC-3' and 5'-TCAGCCTCTGCATGATGTTC-3'; Glyceraldehyde-3-phosphate dehydrogenase, 5'-TGATGGGTGTGAACCACGAG-3' and 5'-GGGCCATCCACAGTCTTCTG -3'.

Statistical Analysis

A logrank test was performed to test difference in survival between MV LPS and MV LPS+L. Difference among groups regarding time courses of mean arterial pressure (MAP) was performed with a two-way ANOVA. D'Agostino-Pearson test was used to test verify normal distribution of all parameters studied. A unpaired Student t-test was performed to evaluate the statistical significance of differences of the following normally distributed parameters (nitrosylated proteins and HNE-protein between MV-LPS and MV LPS+L animals). Differences

between normally distributed parameters of diaphragm KC, plasma IL-10, IL-6, KC and caspase-3 activity regarding were analyzed with one-way ANOVA. Student-Newman-Keuls post hoc testing was used to test the probability level of differences between nominal divided groups. Differences between parameters not normally distributed (iNOS mRNA, diaphragm IL-1beta, IL-10, TNF-alpha, IL-6, plasma IL-1beta and TNF-alpha, MuRF-1 and MAFbx mRNA) were analyzed with one-way ANOVA Kruskal-Wallis and Dunn's post hoc testing. For statistical analysis of cytokine measurements the value of the detection limit was used for samples that did not reach the detection limit. Graphpad prism was used to conduct statistical analysis (Graphpad Software Inc., San Diego, CA). A probability level of $p < 0.05$ was considered significant. All data are presented as mean ± SD.

RESULTS

Animal characteristics

Blood pressure decreased progressively in both groups during 8 hours of mechanical ventilation (Figure 1, $p = 0.09$), despite volume therapy (0,3 ml/h). Bloodgas analysis at the end of the experiment is shown in table 1. Alveolar-arterial (A-a) oxygen gradient was high after 8 hours of mechanical ventilation in both groups (Table 1). Within the 8 hours of mechanical ventilation 30% (n=5) of the animals in the MV LPS group died (respectively 4 hours, 5 hours, 5½ hours and 2 mice after 6 hours after initiation of mechanical ventilation). In the levosimendan treated group, 12% (n=2) of the mice died before the end of planned duration of mechanical ventilation (after 5 and 7 ½ hours of ventilation respectively; $p = 0.2$ between groups). Animals that did not survive until the end of the study were excluded from further biochemical analysis. Accordingly, for biochemical analysis 8 CON mice, 12 MV LPS mice and 14 MV LPS+L mice were included.

Nitrosative- and oxidative stress

To investigate the effects of levosimendan on nitrosative- and oxidative stress in endotoxemic mechanically ventilated mice, diaphragm was analyzed on iNOS expression, protein nitrosylation and HNE-protein. Diaphragm iNOS expression was significantly increased by 274% in LPS exposed ventilated mice ($p < 0.05$ vs CON, Figure 2A). Unexpectedly, levosimendan enhanced the expression of iNOS by 47% (Figure 2A; $p < 0.05$ MV LPS vs MV LPS+L), but decreased diaphragm protein nitrosylation by 10% (Figure 2B/C; MV LPS vs MV LPS+L; $p < 0.05$). Diaphragm HNE-protein concentration was not significantly different between MV LPS animals versus CON animals. Levosimendan decreased the amount of HNE proteins in the diaphragm compared to MV LPS by 13% (Figure 2D/E; MV LPS vs MV LPS+L; $p < 0.05$).

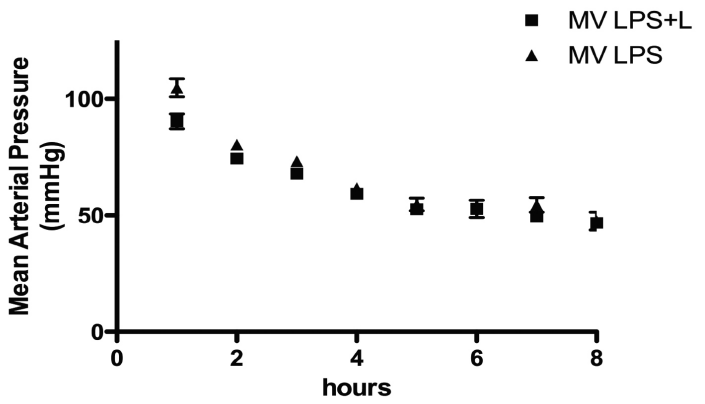

Figure 1 Course of blood MAP during 8 hours of mechanical ventilation.

	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	HCO_3 (mmol/L)	BE (mEq/L)	A-a gradient (mmHg)
MV LPS+L	7.23 ± 0.0	153 ± 11	26 ± 1.3	11 ± 0.8	−16.6 ± 1.0	135 ± 9.4
MV LPS	7.24 ± 0.0	153 ± 14	25 ± 2.5	12 ± 1.0	−15.7 ± 1.3	136 ± 8.3

Table 1 Arterial bloodgas and alveolar-arterial gradient after 8 hours of mechanical ventilation and endotoxemia exposure. Values are mean ± SEM. BE = base excess; $PaCO_2$ = arterial carbon dioxide tension; PaO_2 = arterial oxygen tension; A-a gradient = Alveolar arterial gradient .

Inflammation

In MV LPS mice, proinflammatory cytokines were significantly upregulated in diaphragm muscle and plasma (Figure 3 and Figure 4). Levosimendan did not affect this inflammatory response.

Caspase-3 and E3-ligases

Caspase-3 and E3-ligases, MuRF-1 and MAFbx, were analyzed as measures for muscle proteolysis. No differences in caspase-3 activity and MuRF-1 mRNA were observed between different groups. MAFbx expression was significantly increased in MV LPS and MV LPS+L animals compared to control (CON 27±12; MV LPS 102±35; MV LPS+L 114±47, ratio to GAPDH). Levosimendan did not affect expression of MAFbx in ventilated endotoxemic mice.

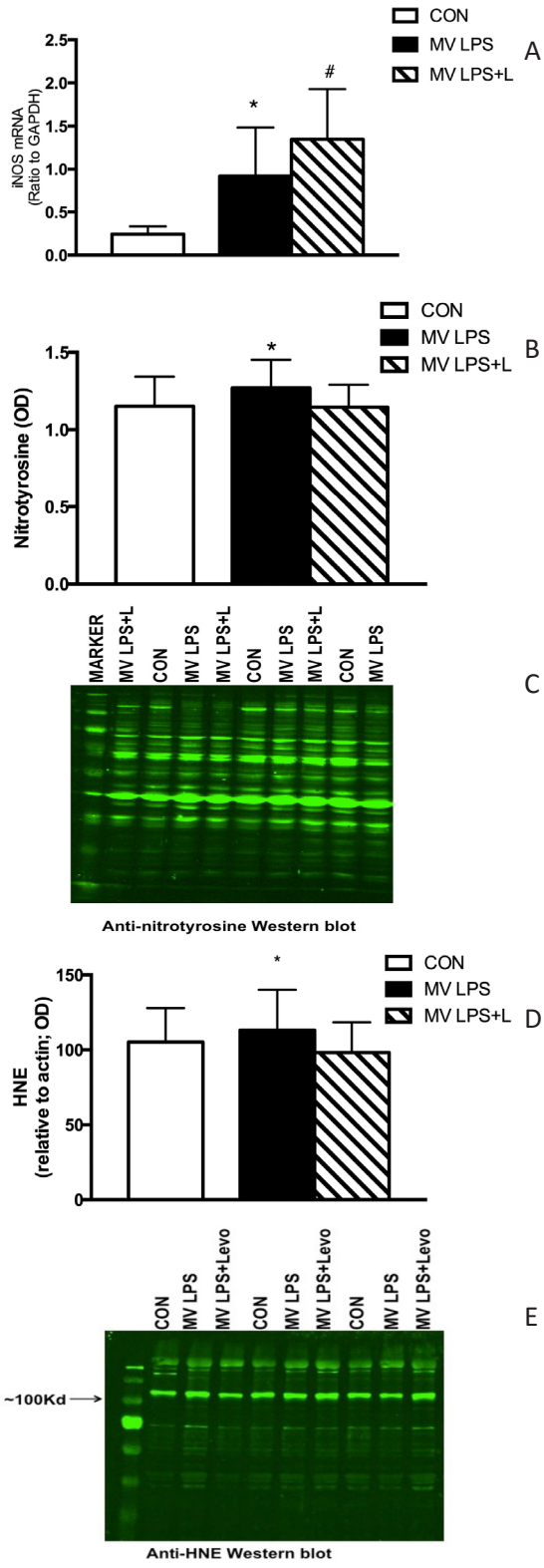

Figure 2 A) Expression of iNOS in diaphragm. iNOS expression was increased in MV LPS versus CON (* $p < 0.05$). iNOS expression was increased in MV LPS+L versus CON and MV LPS (# $p < 0.05$).
B) Nitrotyrosine concentration in diaphragm. Nitrotyrosine concentration was higher in MV LPS from MV LPS+L (* $p < 0.05$).
C) Representative Nitrotyrosine blot.
D) HNE-protein concentration relative to actin concentration in diaphragm. HNE-protein concentration was higher in MV LPS from MV LPS+L (* $p < 0.05$).
E) Representative HNE-protein blot.

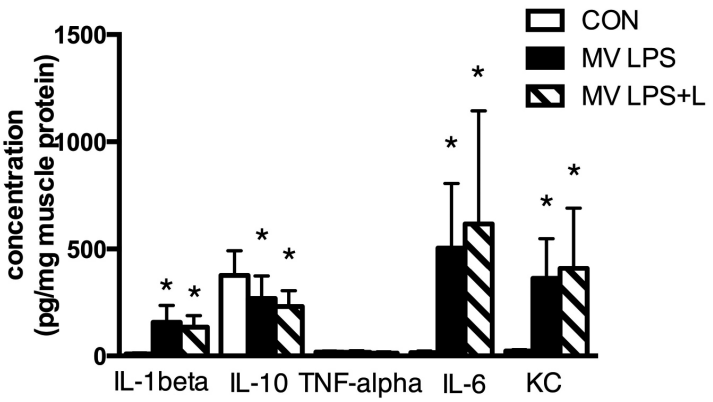

Figure 3 Cytokine levels in diaphragm homogenates. MV LPS and MV LPS+L increased levels of IL-1beta, IL-6, KC and IL-10 (* $p < 0.05$ versus CON).

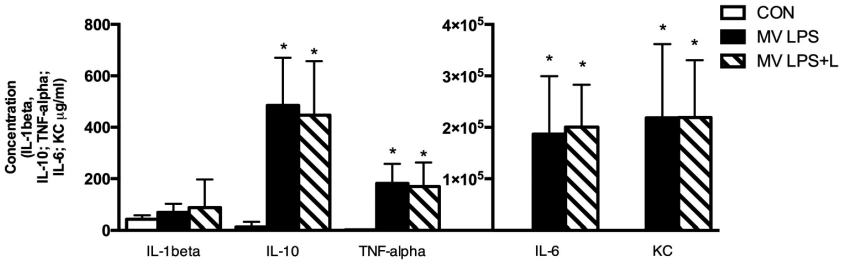

Figure 4 Plasma cytokines. Levels of IL-10 and TNF-alpha, IL-6 and KC were increased in MV LPS and MV LPS+L (* $p < 0.05$ versus CON).

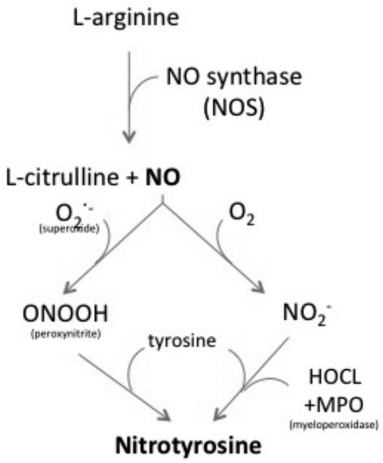

Figure 5 Chemical reactions involved in the formation of nitrotyrosine.

DISCUSSION

The main findings of the current study are that (1) levosimendan decreased protein nitrosylation and markers of oxidative stress in the diaphragm of endotoxemic mechanically ventilated mice. However, (2) levosimendan did not attenuate diaphragmatic and systemic inflammatory response, diaphragm iNOS and E3-ligase expression nor caspase-3 activity.

Effects of endotoxemia and mechanical ventilation on the diaphragm

LPS in rodents is a widely used model to study the effects of endotoxemia on organ function, including respiratory muscles [28-31]. We found increased expression of inducible nitric oxide synthase (iNOS) in the diaphragm of these animals. This is in line with the only previous study that investigated a combination of endotoxemia and mechanical ventilation, reporting that diaphragm weakness was associated with elevated levels of iNOS protein [10]. In addition, in the current study pro-inflammatory cytokines were upregulated in the diaphragm of endotoxemic ventilated animals. This inflammatory response is consistent with earlier experimental models of either mechanical ventilation or endotoxemia [16, 31, 32]. Mechanical ventilation as well as endotoxemia are known to increases proteolysis in the diaphragm, as supported for example by elevated caspase-3 activity and expression of E3-ligases MuRF-1 and MAFbx [5, 33-35]. Expression of MAFbx was also enhanced in our endotoxemic ventilated mice. MuRF-1 expression and caspase-3 activity were not elevated in endotoxemic ventilated mice. This suggests that the proteolytic response to a combination of mechanical ventilation and endotoxemia is weaker than to mechanical ventilation or endotoxemia separately. Reduced proteolysis could preserve diaphragm function. This is supported by findings from a previous study in rats [10], where mechanical ventilation was shown to partly prevent the development of diaphragm dysfunction during endotoxemia. However, it should be acknowledged that in the current study the effect of mechanical ventilation solely on the diaphragm was not evaluated, as this was not required to test the hypotheses of the current study.

It has been shown previously that endotoxemia is associated with pulmonary inflammation and elevated the alveolar-arterial oxygen gradient [36]. In line with those observations, we found in endotoxemic ventilated mice a high A-a gradient (Table 1). The A-a gradient was not assessed in control mice in the current study. We have previously shown that the alveolar-arterial oxygen gradient in healthy mice ventilated for 8 hours was higher than normal, (79 +/- 21 mmHg) [16], but significantly lower than reported in the current study for LPS exposed ventilated mice. This indicates that systemic effects of LPS further impaired oxygen uptake in the endotoxemic ventilated groups of the present study.

Effects of levosimendan

Among its inotropic effects through calcium sensitization, levosimendan has been shown to affect several intracellular pathways involved in oxidative stress and inflammation [21, 23]. In the present study, levosimendan infusion decreased nitrosylated protein levels in ventilated endotoxemic mice (Figure 2B). Peroxynitrite formation is one of the chemical reactions involved in the nitrosylation of tyrosine residues in proteins (Figure 5), [37, 38]. Peroxynitrite is an extremely reactive free radical generated from the reaction between nitric oxide and superoxide [39]. NO is synthesized from l-arginine under influence of the three NOS enzymes, iNOS, eNOS and nNOS [40].

In the current study, levosimendan did not reduce iNOS expression (Figure 2A) in diaphragm of mechanically ventilated endotoxemic animals. This may suggest that levosimendan reduced peroxynitrite formation by lowering superoxide levels. Decreased superoxide levels are expected to be accompanied by a reduction of markers for oxidative stress. In line with that assumption, HNE protein levels (a marker for oxidative stress) were decreased in the diaphragm of levosimendan treated mice (Figure 2D). In accordance with the possible ability of levosimendan to reduce oxidative stress, a recent investigation showed that levosimendan treatment increased protective antioxidant enzyme levels in renal tissues of rats [41]. Independent from the peroxynitrite formation, myeloperoxidases can induce tyrosine nitrosylation (Figure 5) [42]. Interestingly, previous experimental studies have shown that levosimendan reduces myeloperoxidase activity in the heart and spinal cord [43, 44], but this has not been investigated in the diaphragm. Although we did not specifically investigate this pathway, we cannot exclude that levosimendan decreased protein nitrosylation by reducing myeloperoxidase activity.

In the current study, levosimendan did not attenuate the pro-inflammatory response to endotoxemia and mechanical ventilation neither in plasma nor in the diaphragm (Figure 3 & 4). The lack of effect of levosimendan on plasma inflammation after levosimendan exposure is in line with previous studies [45, 46]. In apparent contrast, in experimental sepsis induced by cecal ligation and incision, levosimendan did reduce high plasma levels of IL-1beta [24]. However, in our animal model plasma levels of IL1-beta were not elevated after LPS exposure. Furthermore, levosimendan did not dampen MAFbx expression in endotoxemic ventilated mice. Interestingly, IL-6 is a well-known regulator of MAFbx gene expression [47]. The absence of an effect of levosimendan on plasma and diaphragm IL-6 could be an explanation for the absence of a dampened effect of levosimendan on MAFbx expression. The activation of caspase-3, another essential player in muscle protein degradation, was reduced in the spleen of septic animals treated with levosimendan [24]. Also, in cardiac muscle cells levosimendan could protect

against hydrogen peroxide induced caspase-3 activation [23]. In the present study caspase-3 activity in the diaphragm was unchanged by levosimendan, but also not enhanced by endotoxemia and mechanical ventilation.

Study limitations

The present study has limitations that should be acknowledged. First, the duration of mechanical ventilation and endotoxemia in the present study is relatively short when compared to critically ill patients. It should be noted that mechanical ventilation in mice is challenging, due to limited possibilities of vital function monitoring and the fact that due to very small intravascular volume (~2 ml), no repetitive samples can be withdrawn for blood gas analysis. Nevertheless, in the current study 8 hours mechanical ventilation resulted in modulation of important biochemical pathways in the diaphragm, in line with our previous study shown previously [16]. Therefore, this time period is appropriate to study the effects of levosimendan on activation of inflammatory and proteolytic pathways. A second limitation of our study is that we did not assess the effects of levosimendan on diaphragm muscle function. Previously, we have shown that levosimendan improves diaphragm muscle function, both in vitro and in vivo in healthy subjects, in patients with COPD and animal models for congestive heart failure [18, 19, 48]. We can only speculate that it also will improve diaphragm function in the current model, but this should be confirmed in future studies. Thirdly, as mechanical ventilation of endotoxemic mice is technically challenging, we were reluctant to invasively monitor cardiac output or tissue perfusion, which is of potential interest as levosimendan has both been shown to improve cardiac output and induce vasodilation [20]. Finally, in the present study LPS was used to induce an inflammatory response, LPS administration to animals has been proven to be a predictable model of systemic inflammation [49]. However it should be acknowledged that this is a model for systemic inflammation, and not necessarily reflect the physiological processes observed in human septic shock.

CONCLUSION

In an animal model of endotoxemia and mechanical ventilation, levosimendan decreases markers of oxidative and nitrosative stress. Therefore our data may suggest, that the beneficial effects of levosimendan in clinical trials [20, 50] may partly result from effects beyond calcium sensitization. The current study provides a rationale to investigate such a mechanism in a clinical study. We are currently investigating levosimendan in a randomized clinical trial (NCT01721434) in patients difficult to wean from the ventilator.

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Chapter 7

Strategies to optimize respiratory muscle function in weak ICU patients

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ABSTRACT

Respiratory muscle dysfunction may develop rapidly in critically ill ventilated patients and is associated with increased morbidity, length of ICU stay, costs and mortality. This review briefly discusses the pathophysiology of respiratory muscle dysfunction in ICU patients and will then focus on strategies that prevent the development of muscle weakness, or if weakness has developed how respiratory muscle function may be improved. The authors propose a simple strategy how these strategies can be implemented in clinical care.

INTRODUCTION

Ventilatory failure develops in case of an imbalance in loading and capacity of the respiratory muscle pump. Reduced force output of the respiratory muscles is frequently seen in ventilated critically ill patients [1-4]. In fact, only a brief period of controlled mechanical ventilation (±1-3 days) is associated with development of diaphragm muscle atrophy [5]. Subsequently, Jaber and colleagues [1] demonstrated that after 5-6 days of controlled mechanical ventilation force-generating capacity of the diaphragm was reduced by ±32%. In ICU patients, impaired capacity of the respiratory muscles is accompanied by an increased load due to elevated elastic and resistive forces of the respiratory system. This imbalance in load and capacity of the inspiratory muscles plays an important role in the development of ventilatory failure, for instance during a weaning trial. Respiratory muscle weakness is associated with adverse clinical outcomes, including difficult weaning from mechanical ventilation, increased aspiration risk, increased mortality [6-9], increased risk of ICU / hospital readmission [10] and increased costs [11]. Proposed risk factors for the development of ICU acquired respiratory muscle weakness include disuse due to excessive ventilator support, systemic inflammation (infection, sepsis), neuromuscular blockers and steroids [12-15].

It is reasonable to propose that strategies that aim to improve respiratory muscle function in these patients improve outcome. Indeed, inspiratory muscle training enhances weaning from the ventilator in weak ICU patients [16]. However, today no drugs are approved to enhance respiratory muscle function. This is surprising, as several drugs are available to improve cardiac muscle contractility. However, physiological studies have provided evidence that pharmacological modulation of respiratory muscle contractility is indeed feasible [17, 18]. The aim of the present paper is to discuss (future) strategies that prevent the development of respiratory muscle weakness or restore respiratory muscle function in weak ICU patients. We will focus on interventions that are most likely to be of clinical importance in the near future. First, we will briefly summarize the pathophysiology of ICU acquired respiratory muscle weakness.

Pathophysiology of respiratory muscle weakness in the critically ill

Reduced force output of the respiratory muscles in the critically ill may result from injury at any point between the central respiratory centres and the contractile proteins of diaphragm muscle fibers [12, 14]. In the absence of sedatives, reduced central respiratory drive is unlikely to explain reduced force output of the respiratory muscles in ICU patients [19]. Phrenic nerve neuropathy, as assessed by prolonged phrenic nerve conduction time, has been demonstrated in ICU

patients, indicating that injury of the peripheral nerve may play a role in reduced force output [20].

Contractile dysfunction of the respiratory muscles in ICU patients may result from the loss of muscle mass (atrophy) and / or dysfunction of the remaining contractile proteins. In a landmark paper, Levine and colleagues demonstrated the rapid development of diaphragm muscle atrophy in ventilated brain dead patients [5]. More recently, Hooijman and colleagues performed in depth functional and structural analysis of diaphragm biopsies in critically ill patients on the ventilator [21]. In that study, muscle fiber cross sectional area was reduced by ±25% after an average of 7 days of mechanical ventilation. Interestingly, the diaphragm appeared more susceptible to atrophy compared to other respiratory and non-respiratory muscles [5, 21, 22]. Muscle atrophy is the final result of an imbalance between protein synthesis and degradation. Upregulation of several proteolytic pathways has been demonstrated in the respiratory muscles of ICU patients [21, 23]. For instance, key regulators of the ubiquitin-proteasome pathway are upregulated in the diaphragm of these patients [5, 21]. Other pathways such as lysosomal protein degradation and autophagy may play a role as well (Figure 1) [13, 24]. In addition to enhanced proteolysis, decreased protein synthesis has been reported in the diaphragm of rodents subjected to controlled mechanical ventilation [25, 26]. Also, decreased protein synthesis of non-respiratory skeletal muscles has been reported in critically ill patients [15, 27]. In addition to atrophy, diaphragm weakness may be the result of contractile protein dysfunction. Even when corrected for loss of protein, muscle fibers in ICU patients develop less force. In addition, the sensitivity for calcium of the contractile proteins is reduced [4]. The pathophysiology of contractile protein dysfunction in these patients is incompletely understood, but phosphorylation and oxidative modifications of the sarcomeric proteins appear to play a role [28, 29]. For extensive background on the pathophysiology of muscle dysfunction in the critically ill, we refer to a recent excellent review on this subject [30].

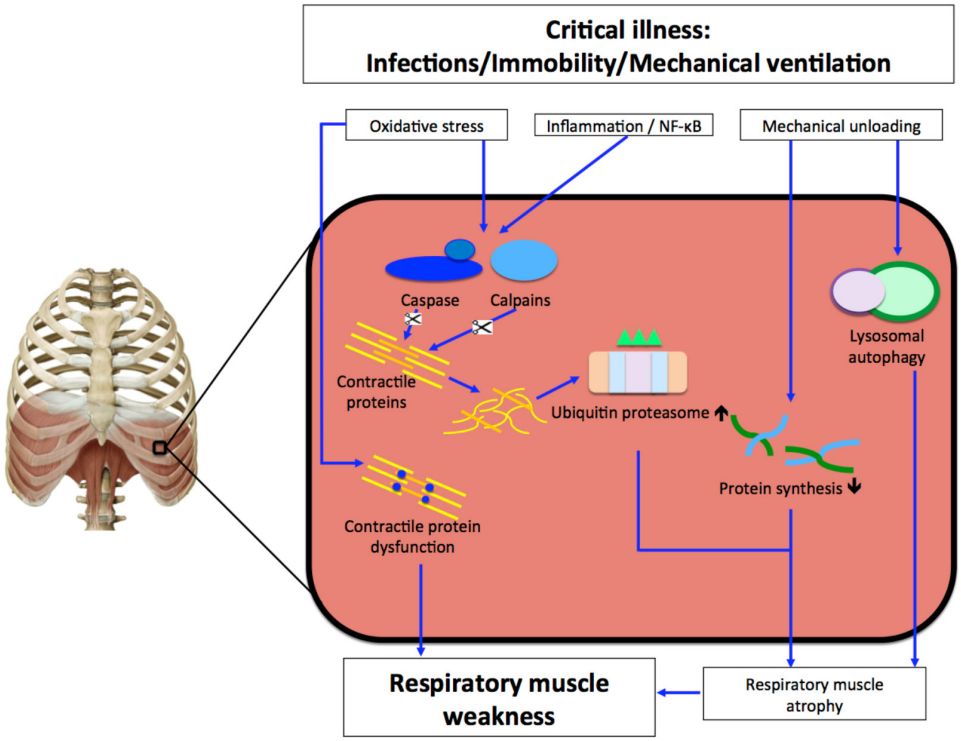

Figure 1 Proposed scheme of pathofysiologic pathways in the development of respiratory muscle weakness during critical illness. Oxidative stress [31], inflammation [32, 33], increase in NF-κB activity [34] and mechanical unloading [5, 21] have been proposed to initiate respiratory muscle weakness. Due to these initiators, contractile protein can become dysfunctional [4], synthesis can be decreased [25, 26], or cause musculair autophagy [24]. Oxidative stress and inflammatory pathways can activate caspases and calpains [31, 35]. Thereby, these pathways can deliver substrate for the ubiquitin-proteasome [5, 21, 36] that further degradates contractile proteins.

In conclusion, the pathophysiology of ICU acquired respiratory muscle weakness involves the activation / inhibition of multiple molecular pathways associated with the protein balance and post-translational modifications of the contractile machinery. Understanding these pathways provides a rationale to evaluate specific interventions to improve respiratory muscle function in these patients.

Evaluation of respiratory muscle function in ICU patients

Evaluation of respiratory muscle function is a challenge, in particular in ventilated ICU patients. However, both for diagnostic purposes and evaluation of the effect of interventions in depth analysis of respiratory muscle function may be

necessary. We will briefly discuss the readily available techniques that are relevant and feasible in clinical practice. For a detailed overview we refer to other review papers [37-39].

Maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP) are relatively simple tests to evaluate global respiratory muscle strength and can be applied to selected ICU patients. MIP and MEP are measured using a handheld pressure device connected to the endotracheal tube or tracheostomy while the patient performs specific manoeuvres. Alternatively, pressures can be assessed using the ventilator by performing an end-expiratory hold manoeuvre. It should be noted that high values exclude clinically significant weakness, but low values are common and may also reflect poor technique or effort [40]. A 20-second end-expiratory occlusion can be performed to obtain more reliable measurements in poorly cooperative patients [41].

Esophageal pressure (Pes) is an estimate of pleural pressure [42], and can be used to calculate the amount of work performed by the respiratory muscles. Simultaneous recording of Pes and gastric pressure (Pga) allows the calculation of transdiaphragmatic pressure (Pdi = Pga - Pes), a specific measure of diaphragm contractility. The latter is useful for close monitoring and evaluation of diaphragm function in difficult-to-wean patients [7], However, acquisition, calculation and interpretation of Pes, Pga and their derived measures are rather complex and therefore not widely accepted in clinical practice.

Ultrasonography is an increasingly popular tool for assessment of diaphragm function, as it is widely available, portable, non-invasive, rather easy to learn and has been validated in ICU patients [43-45]. Visualizing diaphragm excursion during unassisted respiration allows detection of reduced or paradoxal movement. Thickness of the diaphragm muscle at end-expiration is used to detect atrophy [46], and its thickening fraction during inspiration to asses contractile activity [44]. Future fields of application may be detection of patient-ventilator asynchrony [47], and assessment of respiratory workload [48, 49].

Diaphragm electromyography (EMG) reflects the electrical activity and is the gold standard to assess neural respiratory drive. Diaphragm EMG can be recorded best using an esophageal catheter with multiple electrodes. With the introduction of neurally adjusted ventilatory assist (NAVA) (Maquet, Solna, Sweden) [50], the (processed) EMG signal can be obtained continuously in clinical care of ventilated patients. In these patients, diaphragm EMG may be used to monitor respiratory muscle unloading [51] and patient-ventilator interaction [52].

Modulation of contractile activity: Disuse and inspiratory muscle training

A. Prevention of disuse atrophy

Like any other striated muscle, respiratory muscle mass is affected by contractile activity. In fact, the respiratory muscles appear more sensitive to the effects of disuse compared to other striated muscles [5, 21, 22]. In humans, relatively brief periods of diaphragm disuse (< 3 days) due to controlled mechanical ventilation are associated with diaphragm muscle fiber atrophy [5].

Animal studies have demonstrated that mechanical ventilation-induced diaphragm atrophy and dysfunction is less severe in assisted modes of mechanical ventilation [53]. However, 48 hours of muscle relaxants in patients with early severe ARDS, in fact fastened liberation from mechanical ventilation and did not adversely affect peripheral muscle function, virtually excluding a clinically relevant adverse effect of short-term muscle relaxants on respiratory muscle function in the early phase of severe critical illness [54]. Therefore, it appears that under certain conditions (i.e. severe ARDS) controlled mechanical ventilation is preferred to facilitate lung-protective ventilation and this beneficial effect outweighs the possible adverse effects on the respiratory muscles. Nevertheless, it is reasonable to limit the duration of controlled mechanical ventilation and such diaphragm inactivity, to reduce the risk of disuse atrophy. Recently, we showed that assisted ventilation allows lung protective ventilation in selected patients with mild to moderate ARDS [55]. It is important to recognize that ventilator pressure and flow waveforms are unreliable to confirm the presence of respiratory muscle activity [37, 38, 56]. We recommend additional monitoring techniques such as continuous recording of esophageal pressure, diaphragm electrical activity or ultrasound [37, 38, 44, 56]. Although the optimal level of activity for the respiratory muscles is unknown during mechanical ventilation, these monitoring techniques allow us to detect complete inactivity of the muscles.

B. Inspiratory muscle training

In general, training can be instituted to enhance muscle endurance or strength. These types of training require different strategies and have distinct physiological responses. In healthy subjects, respiratory muscle activity is characterized by development of low pressure during the entire life span of a subject. The pressure generated by the inspiratory muscles is only ±5 cmH_2O (5% of maximum inspiratory pressure) to generate a tidal volume of 500 ml, when respiratory compliance is 100 ml/cmH_2O. At first sight, training of the respiratory muscle should therefore be designed to improve endurance. Indeed, in patients difficult to wean from the ventilator progressive weaning trials (T-tube or low pressure support) are frequently instituted as training stimulus. Although reasonable from a physiological perspective, it has never been proven that this strategy indeed improves respiratory muscle endurance.

There are very few circumstances where high inspiratory pressure is required for prolonged periods of time and therefore strength training of the diaphragm seems of limited relevance. However, it has been demonstrated that respiratory effort sensation depends on the maximal inspiratory pressure [57]. In healthy subjects, pharmacological induction of inspiratory muscle weakness increases respiratory effort sensation for the same workload [57] confirming the importance of adequate strength beyond the strength strictly required to generate tidal volume. It has been shown that inspiratory muscle strength training (IMST) improves whole body exercise performance, in particular in less fit subjects [58]. Also, in patients with COPD inspiratory muscle training improves inspiratory muscle strength, total body exercise and reduces dyspnea sensation [59]. Only three randomized studies have reported the effectiveness of IMST in ventilated ICU patients. In the trial by Cader [60], 41 ventilated ICU patients with respiratory muscle weakness were randomized between inspiratory threshold loading and no training intervention. Training consisted of an inspiratory load of 30% maximum inspiratory pressure for 5 minutes, twice a day, seven days a week throughout the weaning period. Maximum inspiratory pressure was significantly increased in the training group (15 ± 3 cmH_2O to 25 ± 4 cmH_2O), but not in the control group (15 ± 2 cmH_2O to 18 ± 2 cmH_2O). The study was underpowered for clinical relevant endpoints, although weaning time was reduced in the training group. In another study, Martin and colleagues [16] randomized 69 ventilator bound patients (mean duration of ventilation at inclusion ±44 days) to inspiratory muscle training or sham training added to endurance training. Strength training consisted of four sets of 6-10 breaths per day with 2 minutes rest between each set. Loading was individualized and set at a level that could just be tolerated by the patient. Inspiratory muscle strength training significantly improved maximum inspiratory pressure (end of training 54cmH^2O in intervention group and 45cmH_2O in sham group, $P<0.05$). Also, successful weaning at day 28 after inclusion was more likely in the intervention group compared to sham (71% versus 47%, $P<0.05$). No adverse events of IMST were reported in these two trails. Finally, Condessa [61] randomized ventilated patients to inspiratory strength training on top of usual care versus usual care only. Each training session consisted of 5 sets of loaded breaths (40% maximum inspiratory pressure), twice a day, seven days a week. In this study, inspiratory strength training significantly increased maximum inspiratory pressure, but did not affect weaning time.

In conclusion, inspiratory muscle training is feasible and appears safe in patients with respiratory muscle weakness difficult to wean from the ventilator. Studies in other patient categories, including COPD, indicate that inspiratory muscle training improves outcome. In our opinion, it is reasonable to add inspiratory muscle strength training to endurance training in stable difficult to wean patients with

confirmed respiratory muscle weakness. Future studies are needed to determine the optimal training protocol and appropriate timing for initiation of inspiratory muscle training.

Antioxidants and nutrition

As outlined above oxidative stress may play a role in the pathophysiology of ICU acquired respiratory muscle weakness. Several studies have evaluated the effects of a variety of antioxidants on respiratory muscle function, including ICU patients. Numerous animal studies have demonstrated that antioxidants attenuate the detrimental effects of controlled mechanical ventilation and / or systemic inflammation on respiratory muscle structure and function [62-68]. In healthy subjects, high dose N-acetylcysteine (150 mg/kg *i.v.*), attenuated diaphragm fatigue induced by an inspiratory resistive load [69]. Today, no study has specifically evaluated the effect of antioxidants on respiratory muscle function in ICU patients. However, some indirect evidence is available. In an open trial, 595 critically ill surgery or trauma patients were randomized between antioxidants suppletion (alpha-tocopherol and ascorbic acid) and standard care or standard care only [70]. Patients in the antioxidant group spent less time on the ventilator (3.7 versus 4.6 days; $P<0.05$). It should be noted that patients in this study were young (38±15 yrs) and total ventilation time was rather short. Therefore, it is questionable whether the beneficial effects of antioxidants were the result of improved respiratory muscle function. Heyland studied the effects of antioxidants and glutamine in a heterogeneous ICU population (N=1223) [71]. Patients were divided in 4 groups: placebo, glutamine (0,35 gram/kg/24hours of body weight intravenously), antioxidants (selenium, zinc, beta carotene, vitamin E and vitamin C), or antioxidants + glutamine. Again, respiratory muscle function was not specifically evaluated, but no difference in duration of mechanical ventilation was observed among the four groups.

Today, only one study has evaluated the effects of two nutritional strategies on skeletal muscle structure and function in critically ill patients [72]. This study was a planned subanalysis of the EPaNIC trial, that compared the effects of tolerating macronutrient deficiency versus early parenteral nutrition on skeletal muscle structure and function [73]. In that study, patients were randomized between early (within 2 days of ICU admission) versus late (<8 days after ICU admission) parenteral nutrition to prevent macronutrient deficiency. Skeletal muscle strength was assessed in 600 ICU patients using the Medical Research Council sum score. Weakness occurred less often in late versus early parenteral nutrition group (34% vs 43% P=0.030). Compared to healthy subjects, muscle fibers exhibited atrophy, but not significantly different between the early and late parenteral nutrition group. However, markers for autophagosome formation were significantly

higher in the late parenteral nutrition group. This marks that autophagy plays an important role in protein turnover next to other effects to provide substrate for recycling. In conclusion, tolerating macronutrient deficit in the first week after ICU admission is not associated with the development of muscle fiber atrophy, but surprisingly appears to improve muscle contractility, together with activation of autophagy.

Although the effect of high dose antioxidant administration on respiratory muscle structure and function is encouraging in animals models, no data support the routine administration of high dose antioxidants or other specific feeding strategies on respiratory muscle function in the critically ill ventilated patient. Therefore, we do not recommend routine suppletion of antioxidants to the weak ICU patients in order to improve respiratory muscle function.

Improve respiratory muscle protein content: anabolics

Loss of muscle mass plays an important role in the development of ICU acquired respiratory muscle weakness [5, 21] and is the result of an imbalance between protein synthesis and degradation. Aiming to restore the protein imbalance is a reasonable approach in these patients. First, inhibitors of proteolysis have been used in animal models [74, 75], and indeed improve respiratory muscle mass. However, these agents are relatively toxic and not approved to enhance muscle mass in humans.

Second, protein synthesis can be increased with anabolic hormones. Anabolic hormones and its analogues have been used to enhance skeletal muscle mass under a variety of conditions (for review: ref. [76]). We will focus on the effects of endogenous and exogenous anabolic hormones on the respiratory muscles. The most important endogenous anabolic hormones are growth hormone (GH), insulin-like growth factor-1 (IGF-1), insulin and the anabolic steroid testosterone and its analogues.

The effects of GH on skeletal muscle have been reviewed recently [77]. GH enhances production of the powerful anabolic hormone IGF-1 in the liver, but GH has direct anabolic effects on skeletal muscle as well. The effect of GH / IGF-1 or its exogenous analogues administration on respiratory muscle function in critically ill patients has not been studied. However, Takala reported the effects of recombinant-GH (Genotropin) administration in critically ill ICU patients relatively early during ICU stay (inclusion between day 5 and 7) in two placebo controlled trials [78]. Surprisingly, both trials demonstrated significantly increased mortality in rGH treated patients (47% vs. 25% in the finish study and 61% vs. 26% in the multinational study). The mechanisms for increased mortality are incompletely understood, but modulation of immune function may play a role. Although there is still a good rationale for GH / IGF-1 in *stable* ICU patients with respiratory

muscle weakness and difficulty to wean from mechanical ventilation, the safety and efficacy needs to be established.

No data are available concerning the effects of anabolic steroids on respiratory muscle function in critically ill patients. However, Schols [79] and colleagues reported the effects of the exogenous anabolic steroid nadrolone in patients with COPD during an eight weeks pulmonary rehabilitation program. Patients (N=217) were randomized between placebo, placebo with high caloric feeding and nadrolone with high caloric feeding. They found that nandrolone together with high caloric feeding, significantly improved inspiratory muscle strength. These findings were more or less confirmed in a later trial by the same group [80]. Although case series report the use of anabolic steroids in difficult to wean patients [81], no randomized studies have evaluated the safety and efficacy of anabolic steroids in ICU patients with respiratory muscle weakness.

Insulin has anabolic properties as well and interestingly intensive insulin therapy versus conventional insulin therapy in critically ill patients reduces the duration of mechanical ventilation, although it does not affect ICU length of stay [82-84]. The beneficial effect of intensive insulin therapy was probably the result from reduced incidence of critical illness polyneuropathie.

In conclusion, enhancing protein synthesis is a potentially effective strategy to improve respiratory muscle function in patients with ICU acquired muscle weakness. GH is unattractive as it has been associated with increased mortality in ICU patients [78]. Given the encouraging studies on respiratory muscles in COPD, nandrolone would be the most reasonable anabolic hormone to improve respiratory muscle function in selected stable difficult to wean patients.

Positive inotropes

In addition to the loss of contractile protein (atrophy), dysfunction of the remaining muscle fibers has been demonstrated in critically ill patients [4]. Accordingly, optimizing contractility using positive inotropes seems a reasonable approach in these patients. ß-adrenoreceptor agonists indeed exhibit a direct positive inotropic effect on the diaphragm muscle in vitro by increasing intracellular calcium influx [85, 86]. The effects of β2 adrenoreceptor agonists on respiratory muscle function in vivo is controversial [87, 88]. Albuterol (p.o.) did not affect fatigability of the diaphragm in healthy subjects [88]. However, fenoterol (p.o.) delayed the development of diaphragm fatigue in healthy volunteers subjected to inspiratory loading [87]. In mechanically ventilated COPD patients with respiratory failure, respiratory muscle function significantly improved after dopamine infusion probably by augmentation of diaphragm blood flow and improved cardiac output [89].

Muscle fibers isolated from the diaphragm of ICU patients display decreased maximal force generating capacity, indicating intrinsic muscle weakness [21]. In fast-twitch diaphragm fibers the reduction of sub-maximal force generation even exceeds the loss of maximal force generating capacity [4]. This implies that these fibers require more calcium to generate normal force levels, i.e. their sensitivity to calcium is reduced ("reduced calcium sensitivity"). Calcium sensitizers have been developed to treat similar pathology of cardiac muscle [90, 91]. Currently, levosimendan is the only calcium sensitizer approved for use in humans (>50 countries worldwide). Experimental studies have shown that levosimendan also improves calcium sensitivity of diaphragm muscle fibers [17, 92]. Moreover, a recent double-blind, randomized, crossover study demonstrated that administration of levosimendan improved neuromechanical efficiency by >20% and prevented contractile fatigue during diaphragm loading task in healthy subjects [18]. Therefore, levosimendan is a promising drug to improve respiratory muscle function in ICU patients. A current randomized clinical trial (ClinicalTrials.gov identifier NCT01721434) is investigating whether levosimendan indeed facilitates liberation from the ventilator. Levosimendan is generally well tolerated by patients, hypotension is the most observed side effect, but is not common when the treatment remains within the recommended dose, or can easily be counteracted by low dose norepinephrine [93]. In contrast to levosimendan, the effectiveness of other calcium sensitizers has so far only been studied in vitro. For example, exposure to EMD 57033 partially restored calcium sensitivity in diaphragm fibers isolated from piglets after 5 days of mechanical ventilation [94]. Furthermore, in diaphragm fibers from critically ill patients, CK-2066260 completely restores calcium sensitivity [4]. These findings underscore the therapeutic potential of calcium sensitizers and the necessity to evaluate in vivo effects in clinical trials.

Taken together the evidence for improvement of respiratory muscle function with β2 adrenoreceptor agonists is at least controversial. In addition, the inotropic effect of β adrenoreceptor agonists is mediated through enhanced intracellular calcium concentration, which increases muscular energy expenditure and as such CO_2 production. Therefore, β receptor agonists are not very attractive in the critically ill patient. In contrast, calcium sensitizers might very well exert an energetically beneficial effect on diaphragm work [17]. When less calcium is needed to maintain force generation, muscle work becomes more efficient [18] and potentially improve respiratory muscle contractility in critically ill patients.

Future developments

I. Modulation of inflammation

Activation of pro-inflammatory pathways is associated with respiratory muscle weakness [33, 95-97]. Therefore, modulation of the inflammatory response to combat respiratory muscle dysfunction has been scope of interest in recent experimental studies [32, 33, 98]. We demonstrated that interleukin (IL)-6 plays an important role in the loss of contractile proteins in muscle fibers exposed to plasma from septic shock patients [33]. However, diaphragm fiber atrophy due to disuse in brain death patients was not associated with upregulation of Il-6 [5]. IL-10 exhibits anti-inflammatory properties [99]. In a murine model of Pseudomonas aeruginosa pneumonia, diaphragm dysfunction was attenuated after experimental IL-10 expression [99]. During critical illness with subsequent inflammatory status, NF-κB is the key factor for transcription of several cytokines [100]. Recently, evidence was found that inhibition of NF-κB in endotoxemic mice protects against diaphragm muscle weakness, probably due to decreased generation of pro-inflammatory cytokines [98]. Proteolytic pathways can be activated through Toll Like Receptor (TLR)-4, present in muscle plasma membrane [32]. TLR's are essential receptors in recognizing microbes and initiate an inflammatory immune response [101]. In TLR-4 knockout mice, loss of diaphragm contractile protein associated with controlled mechanical ventilation was attenuated compared to wild type mice [32]. In a large clinical trial the TLR-4 antagonist eritoran did however not improve outcome in patients with severe sepsis or septic shock (±80% on mechanical ventilation) [102]. Nevertheless, neither skeletal muscle function, nor duration of mechanical ventilation was assessed in this trial.

Recently, growth and differentiation factor-15 (GDF15) was recognised as a possible factor for inducing skeletal muscle atrophy during critical illness [103, 104]. Bloch and colleagues reported increased plasma concentration of GDF 15 and enhanced expression of GDF-15 in muscle biopsies of ICU patients compared to patients undergoing elective surgery. Furthermore, exposure of skeletal muscle cells to GDF-15 increased expression of muscle related atrophic genes [104]. However, today no clinical data are available to demonstrate if downregulating activity of GDF-15 protects against skeletal muscle for wasting in ICU patients.

Traditionally, steroids are associated with myopathy, atrophy and dysfunction of the respiratory muscles [105-107]. However, the final effects appear to be dose and time dependent, at least in experimental studies. For instance, Maes demonstrated that "low dose" (5mg/kg) methylprednisolone exacerbated ventilator-induced diaphragm dysfunction, whereas high dose (30mg/kg) protected against the deleterious effects of controlled mechanical ventilation on diaphragm function [108]. In ICU patients the effect of steroids on muscle function are conflicting [109-111]. However, no study has prospectively evaluated

the effects of corticosteroids on respiratory muscle function in ventilated ICU patients.
In conclusion, despite the encouraging data that modulation of inflammation improves respiratory muscle function in animals, data in human are scarce, and if present disappointing.

II. Modulation of proteolytic pathways

Since activation of proteolytic systems plays a key role in the development of respiratory muscle dysfunction during critical illness, several experimental studies have investigated the effect of specific inhibitors. In an animal model of endotoxemia, three different specific proteasome inhibitors (MG132, epoxomicin and bortezomib) reduced diaphragm proteolysis, but none of these could prevent the development of contractile dysfunction [36]. Similarly, in rats exposed to 12 hours of mechanical ventilation epoxomicin inhibited proteasome activity, but did not affect diaphragm contractile function or protein content [112]. However, in rats exposed to 24hrs of mechanical ventilation, bortezomib treatment partially prevented the reduction of diaphragm force and atrophy [113]. These small positive effects were probably mediated by the ability of bortezomib to indirectly reduce caspase-3 activity [74]. Proteasomes are only able to process myofilaments that have been cleaved from the sarcomere by enzymes like caspases and calpains [114]. Accordingly, inhibition of the proteasome alone is not expected to have substantial effects on muscle function, as this would only leave the muscle cell with unprocessed, but cleaved myofilaments. Furthermore, considering the basic housekeeping cell functions of the proteasome it is no surprise that the clinical application of bortezomib is accompanied by serious toxic adverse events, like cytopenia and peripheral neuropathy [115]. Thus, pathways upstream of the proteasomes are more likely to be effective therapeutic targets than the proteasome itself. Indeed, inhibition of either caspases or calpains with specific experimental inhibitors attenuates the development of diaphragm atrophy and dysfunction in animal models of prolonged mechanical ventilation [63, 116, 117] and sepsis [118, 119]. Upstream regulators of caspases also seem to be potential therapeutic targets in sepsis induced diaphragm dysfunction. For example, inhibition of either Jun-N-terminal Kinase (JNK) [120], double stranded RNA-dependent protein kinase (PKR) [121] or p38 Mitogen-activated protein kinase (MAPK) [122] modulated cytokine induced caspase activation and prevent the development of diaphragm weakness in mice exposed to endotoxin. Unfortunately, these experimental inhibitors have so far only been investigated in animals. A recent study reported that beta-hydroxy-beta methylbutyrate (HMB), a metabolite of the amino acid leucine, can also block LPS induced activation of PKR and caspase-3 and prevent diaphragm weakness to occur [123]. This study was

performed in mice, but using comparable dosages HMB has previously shown to be a safe and effective agent that can help to improve skeletal muscle function in elderly and patients with cancer [124, 125]. Finally, although several compounds targeting proteolytic pathways upstream of the proteasome have a high potential to prevent the development of diaphragm weakness, this does not necessarily imply that these agents can also improve function of the weakened diaphragm. Nevertheless modulation of the proteolytic system is a potentially interesting target to modulate loss of respiratory muscle function due to controlled mechanical ventilation.

Clinical implications and conclusion

Weakness of the respiratory muscles frequently develops in the ICU patient and is associated with adverse outcome including prolonged mechanical ventilation. Despite the high incidence and clinical impact of ICU acquired respiratory muscle dysfunction, no specific preventive or therapeutic interventions have been tested in large randomized controlled trials. Therefore, we should rely on interventions that seem reasonable from a physiological perspective or are supported by small clinical studies. As pointed out in 2, interventions could be subdivided into three categories: prevention of respiratory muscle dysfunction, secondly therapeutic strategies that aim to improve respiratory muscle function and finally so-called rescue interventions that should only be applied in exceptional cases and only after discussion with the patient or primary decision makers.

Preventive strategies should limit development of disuse atrophy and muscle damage associated with patient-ventilator asynchrony [126]. We suggest using techniques that monitor diaphragm muscle function [37, 38] to confirm a physiologically acceptable level of diaphragm contractility and allow the clinician to optimize ventilator settings in order to improve patient-ventilator interaction. Drugs with potential side effects on skeletal muscle should be avoided.

Once ICU acquired weakness has developed, a combined program of respiratory muscle endurance training and strength training should be considered. Endurance training can be instituted using progressive weaning trials and strength training by using a device for variable inspiratory threshold loading connected to the endotracheal tube [16].

In patients with persistent weakness of the respiratory muscles that prevents unassisted breathing more controversial interventions could be considered, including anabolics [79] or growth hormone [81]. Using respiratory muscle positive inotropes seems attractive and promising for the near future (NCT01721434). Indeed, recent studies confirm the positive inotropic effects of levosimendan on the diaphragm muscle [18] and its safety profile in critically ill patients is well known [90]. However, its use would still be off label and therefore not routinely recommended.

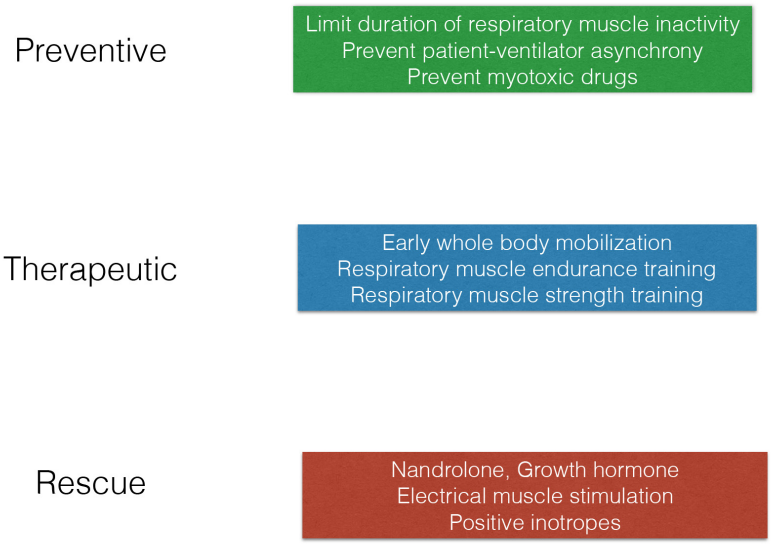

Figure 2 Three groups of interventions to counteract respiratory muscle weakness during critical illness.

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Chapter 8

Summary & Discussion

SUMMARY

Respiratory muscle weakness commonly occurs in the critically ill patient and is strongly associated with increased morbidity and mortality. It has been established that fiber atrophy, decreased protein synthesis and dysfunction of the remaining contractile proteins underlies respiratory muscle weakness in critically ill patients. However, the underlying pathophysiology is largely unknown. The studies presented in this thesis were designed to improve our understanding of how respiratory muscle weakness develops in the critically ill. Also, specific therapeutic strategies to counteract respiratory muscle weakness following mechanical ventilation were explored.

The study presented in ***chapter 2*** demonstrates that 18 hours of mechanical ventilation in rats decreases force-generating capacity of diaphragm muscle fibers. Loss in force is accompanied by a reduction of myosin heavy chain concentration in these muscle fibers. In addition to active force, passive force in muscle fibers can be measured when stretching the muscle fiber. Passive elastic properties of skeletal muscles are indispensible for structural stability and optimal active force generation. Passive force generation upon stretch was significantly reduced in fibers from mechanically ventilated rats. However, titin content - the major protein responsible for passive elastic properties of skeletal muscle - was not different among ventilated and control animals. Dephosphorylation of titin by phosphatase-1 decreased passive force generation upon stretch in diaphragm fibers from control, but not from ventilated rats. This specifies that the effects of mechanical ventilation on passive force reduction can be mimicked by dephosphorylating the elastic protein titin. This indicates that the phosphorylation state of titin in diaphragm fibers from mechanically ventilated animals is already low. We conclude therefore that mechanical ventilation decreases the phosphorylation status of titin in diaphragm fibers, which results in reduced stiffness. Our data are of importance, as we demonstrate that respiratory muscle weakness after mechanical ventilation is accompanied by an impaired active and passive force generation of the respiratory muscles. Intact passive contractile properties of the muscle are assumed to be important to maintain structural integrity of the muscle fiber. Interestingly, mechanical ventilation did not affect active or passive force generation in the soleus muscle, indicating that respiratory muscles are more prone to the deleterious effects of disuse.

Several studies suggest that activation of inflammatory pathways play a role in the development of atrophy and contribute to respiratory muscle weakness in the critically ill. Indeed activation of inflammation has been demonstrated

in the respiratory muscles of animal models with endotoxemia. However, it is unknown if inflammation in the muscle is secondary to systemic inflammation or is the result of a local inflammatory response. Therefore, in ***chapter 3*** it was investigated whether plasma derived from patients with septic shock can induce an atrophic response in skeletal myotubes, supporting the hypothesis that systemic inflammation in these patients contributes to muscle atrophy. Myosin content was ~25% lower in skeletal myotubes exposed to plasma from septic shock patients than in myotubes exposed to plasma of healthy controls. In myotubes exposed to septic shock plasma the amount of ubiquitinated myosin was increased and this increase was accompanied by an increased expression of E3 ligases MuRF-1 and MAFbx in these myotubes. In myotubes exposed to septic shock plasma, we found evidence for activation of inflammation, as demonstrated for instance by activation of NF-κB, an important transcription factor for inflammatory cytokines. Furthermore, elevated levels of IL-6 in plasma of septic shock patients were associated with the severity of myosin loss in myotubes. Interestingly, addition of anti-IL6 to plasma from septic shock patients only partly prevented the loss of myosin in these myotubes. These data indicate that plasma from septic shock patients induces skeletal muscle atrophy by activation of inflammatory pathways, with a central role for IL-6. These data support a role for systemic inflammation in the development of respiratory muscle atrophy in the critically ill.

In ***chapter 4***, the role of toll like receptor4 (TLR4) in the development of atrophy due to controlled mechanical ventilation was investigated. Also, inflammatory pathways were explored upon mechanical ventilation. To this end, wild-type and TLR4 knock-out mice were subjected to controlled mechanical ventilation for 8 hours. In wild-type mice mechanical ventilation resulted in a significant decrease of diaphragm myosin concentration. In contrast, mechanical ventilation did not affect myosin concentration in TLR4 knock-out mice. The pro-inflammatory cytokines IL-6 and keratinocyte-derived chemokine were significantly increased in diaphragm of ventilated wild-type mice, but not in TLR4 knock-out mice. Moreover, light chain 3B-II, a marker of autophagy, was upregulated after mechanical ventilation in the diaphragm of wild-type, but not TLR4 knock-out animals. This study demonstrates that TLR4 signalling plays a role in the development of respiratory muscle atrophy, probably by enhancing inflammatory pathways and activation of lysosomal autophagy.

Hypercapnic acidosis is an accepted side effect of lung-protective ventilation in patients with ARDS. Besides being a side effect of lung-protective ventilation, hypercapnic acidosis itself may exert beneficial effects, for instance by attenuating the systemic and pulmonary inflammatory response. As we demonstrated

that activation of inflammatory pathways may play a role in respiratory muscle atrophy (chapter 3 and 4), we decided to investigate in ***chapter 5*** the effects of hypercapnia on respiratory muscle atrophy and function during controlled mechanical ventilation. Healthy rats were subjected to controlled mechanical ventilation in the absence or presence of hypercapnia. We found that diaphragm muscle myosin concentration was decreased in ventilated control animals, but not animals subjected to hypercapnia. In addition, pro-inflammatory cytokines were significantly upregulated in ventilated control, but not in hypercapnic ventilated animals. Force-generating capacity was decreased in diaphragm single fibers of ventilated animals, and unexpectedly also, although to a lesser extent, in hypercapnic ventilated animals. Since hypercapnia protected respiratory muscles against deleterious effects of myosin loss, probably by a decrease in proteasome activity, during mechanical ventilation but not against loss of muscle function, we investigated involvement of reversible protein modifications by oxidative agents. Single fibers were incubated with antioxidant dithiothreitol and contractility was assessed. Dithiothreitol restored contractility in diaphragm single fibers of hypercapnic ventilated animals but not in control ventilated animals. This indicates that oxidative protein modifications play a role in loss of force induced by controlled mechanical ventilation under hypercapnic conditions. This study indicates that hypercapnia partly protects respiratory muscle to the deleterious effects of mechanical ventilation.

The cardiac inotrope levosimendan improves cardiac muscle contractility by improved sensitivity of troponin for calcium. However, some studies indicate that levosimendan has anti-inflammatory and anti-oxidative properties as well. Since we found an important role for inflammatory pathways in the development of respiratory muscle weakness and atrophy during mechanical ventilation in chapter 3, 4 and 5, we investigated in ***chapter 6*** if treatment with levosimendan can modulate inflammatory and oxidative pathways in respiratory muscle. Mice were exposed to lipopolysaccharide (LPS) to induce a systemic inflammatory response. Next, animals were divided in two groups: placebo and levosimendan. In the diaphragm of mice treated with levosimendan we found a reduction in nitrosylated proteins and also a reduction in markers for oxidative stress. In contrast to earlier studies by other groups, we did not find an effect of levosimendan on pro-inflammatory cytokines. This study shows that levosimendan may protect against nitrosative stress and oxidative stress in the respiratory muscles of endotoxemic mice, but does not modulate the inflammatory response.

DISCUSSION

Respiratory muscle atrophy

Mechanical ventilation is a life saving intervention in patients with acute respiratory failure, but comes with recognized side effects including ventilator-induced lung injury [1], and respiratory muscle dysfunction [2-5]. The current thesis focuses on the effects of mechanical ventilation on the respiratory muscles. Today, the pathophysiology of ventilator-induced respiratory muscle dysfunction is incompletely understood [3, 5, 6]. Studies in the current thesis report that the cross-sectional area of diaphragm fibers was reduced after 18 hours of controlled mechanical ventilation (*chapter 2).* Besides a reduction in cross-sectional area, we found a reduced myosin heavy chain concentration in rats (*chapter 2)* and mice (*chapter 4)* subjected to controlled mechanical ventilation for 18 and 8 hours respectively. These results are in line with previous findings in both humans and rodents [2, 7-9]. In a landmark paper, Levine et al. reported that 18 – 69 hours of controlled mechanical ventilation in brain death patients resulted in respiratory muscle atrophy [2]. That paper indicates that respiratory muscle atrophy develops rapidly in humans when the diaphragm is inactive. In the current thesis, the concentration of myosin was reduced in fibers of ventilated animals, which indicates that the severity of the contractile protein loss exceeds the degree of fiber atrophy (*chapter 2)*. Ultimately, muscle atrophy is the result of a disbalance between proteolysis and protein synthesis [10-12]. The ubiquitin-proteasome pathway appears to play a key role in enhanced proteolysis in the diaphragm during mechanical ventilation and inflammation [2, 3, 12-14]. Briefly, this pathway is characterized by activation of two key enzymes, the E3-ligases MuRF-1 and MAFbx. These enzymes mark proteins for degradation by the proteasome by attachment of a ubiquitin protein [15]. Recent observations in mechanically ventilated critical ill patients confirm an important role for the ubiquitin-proteasome pathway [3]. In that study diaphragm biopsies of ICU patients showed enhanced activity of the ubiquitin-proteasome pathway. This was accompanied by signs of diaphragm atrophy and decrease in diaphragm muscle fiber force. In the current thesis, circulating ligands in plasma of septic shock patients induced atrophy in cultured skeletal myotubes (*chapter 3*). The loss of contractile protein was accompanied by increased expression of the E3 ligases MuRF-1 and MAFbx and increased concentrations of ubiquitinated myosin. Activation of MuRF-1 can be regulated by transcription factor NFκB [16]. Indeed, plasma of septic shock patients enhanced activity of NFκB in cultured myotubes (*chapter 3*). To conclude, plasma of septic shock patients induces atrophy in skeletal myotubes accompanied by activation of the ubiquitin-proteasome pathway and inflammatory pathways (*chapter 3*). This is of importance, since it uncovers that ligands in plasma of septic patients

activate atrophic pathways in "healthy" cultured muscle. Based on these data it can be hypothesized that circulating factors in plasma of septic shock patients induce muscle atrophy in vivo and such induce muscle weakness in these patients. Further research should identify the nature of these specific factors and on strategies that inactivate these atrophic factors.

Role of TLR4

Toll-like receptors (TLR's) are essential in host defence by recognizing specific ligands, including microbial components, but also proteins that are released by damaged tissue [17]. The expression of the subtype TLR4 has been demonstrated in skeletal myotubes and diaphragm tissue [18]. In this thesis the role of TLR4 on the development of ventilator-induced diaphragm atrophy was studied (*chapter 4*). TLR4 deficiency attenuated the development of diaphragm atrophy induced by 8 hours of mechanical ventilation in mice. Previously, activation of TLR4 in diaphragm by its specific ligand lipopolysaccharide (LPS), increased expression of pro-inflammatory cytokines and impaired diaphragm function [19, 20]. TLR4 receptor activation by LPS can result in downstream activation of both the ubiquitin-proteasome pathway and the lysosomal-autophagy pathways in skeletal muscles [21]. In this thesis activation of ubiquitin-proteasome was not different between ventilated wild-type animals and TLR4 deficient animals, indicating that in this model the ubiquitin-proteasome has no prominent role in the development of atrophy. This is in apparent contrast with previous studies that did show an association between mechanical ventilation and induction of diaphragm atrophy through activation of the ubiquitin-proteasome in ventilated humans [2, 3] and rodents [12, 22, 23]. Therefore, we cannot exclude for a role of the ubiquitin-proteasome pathway under every condition in ventilator-induced diaphragm atrophy, in particular after longer duration of mechanical ventilation. In this thesis, we found that autophagy marker LC3B-II was increased in ventilated wild-type animals but not in TLR4 deficient animals (*chapter 4*). Therefore, these data suggest that loss of contractile protein through TLR4 signaling results from activation of the lysosomal autophagy pathway. These data are supported by previous work of Doyle et al, demonstrating that TLR4 agonist LPS, induces lysosomal autophagy in skeletal muscle cells [21]. Also, in ventilated humans the lysosomal autophagy pathway is activated and associated with respiratory muscle atrophy [24]. In conclusion, mechanical ventilation induced loss of contractile proteins may act through activation of the TLR4. Interestingly, TLR4 ligands can increase autophagy in muscle. Therefore, this thesis provides a rationale to further investigate if blocking the TLR4 pathway would benefit the ventilated patient in counteracting respiratory muscle weakness.

Diaphragm function and mechanical ventilation

It is generally accepted that controlled mechanical ventilation impairs force-generating capacity of the diaphragm in animal models and in humans [4, 5, 25, 26]. Reduction in force generating capacity can theoretically result from either a loss of contractile proteins or contractile protein dysfunction. Although atrophy has been demonstrated in the diaphragm of ventilated animals, the role of contractile protein dysfunction is largely unknown. The current thesis describes studies evaluating the force-generating capacity of permeabilized diaphragm fibers (*chapter 2*). Permeabilized fibers are an excellent model to study contractile protein function, as activation does not depend on membrane characteristics or calcium handling. We found that mechanical ventilation reduced force generation in these fibers in proportion to the reduction in cross-sectional area of these fibers (chapter 2). This indicates that remaining contractile proteins have normal force generating capacity. This is however in apparent contrast with other studies that report reduction in force generating capacity of the muscle fibers, even after correction for cross- sectional area [26-28]. For instance, a recent study among a heterogeneous group of critically ill patient revealed that force decrease in diaphragm fibers was partly due to atrophy, but also resulted from dysfunction of the remaining contractile proteins [4]. It should be noted that in critically ill patients a variety of cofactors may play a role besides mechanical ventilation, including administration of certain drugs and infection [9, 29]. Recently, Doorduin et al. demonstrated that the calcium sensitizer levosimendan improves respiratory muscle function in healthy volunteers [30]. In addition, the troponin activator CK-2066260 enhances strength in diaphragm fibers of critically ill patients [4]. To conclude, this thesis clearly demonstrates that mechanical ventilation induces loss of contractile protein in the respiratory muscles resulting in a decrease of force generating capacity of the muscle, but in contract to studies in humans we did not find evidence for contractile protein dysfunction. However, in humans and in particular in the critically ill patient other factors, like for example sepsis and drugs may impair function of the remaining contractile proteins. Therefore, further research for potential strategies to counteract respiratory muscle weakness in the critical ill should not solely focus on dampening atrophic responses but should also evaluate strategies that enhance contractile protein function. Recent and on-going studies with a calcium sensitizers or troponin activator are therefore of importance.

Role of Hypercapnia

In critically ill patients, hypercapnia develops frequently as a consequence of low tidal volume ventilation. In this thesis we investigated the effects of hypercapnic acidosis on the respiratory muscles (*chapter 5*). We found that hypercapnia largely

protected the diaphragm against deleterious effects of mechanical ventilation by maintaining myosin concentration and attenuating the development of atrophy. Therefore it was unexpected that hypercapnia did not preserve contractility in our study. Previous studies have reported that mechanical ventilation under hypercapnic conditions protects against the development of weakness [31]. The data reported in the current thesis indicate that in hypercapnic ventilated animals contractile protein dysfunction developed as the result of posttranslational modification of proteins. Indeed, the reducing agent dithiothreitol restored diaphragm function in diaphragm fibers of hypercapnic ventilated animals, confirming that oxidative contractile protein modification plays an important role in mechanical ventilation under hypercapnic conditions. Indeed, CO_2 can promote generation of oxidants in diaphragm muscle [32]. Our data indicate that further studies should focus in the combined effects of hypercapnia and antioxidants on diaphragm function during mechanical ventilation.

Diaphragm passive force

Passive elastic properties are essential for structural stability and optimal active function of muscle fibers [33]. The protein titin is the key determinant of passive elastic properties in skeletal muscle fibers [34]. Mechanical ventilation reduces passive function of diaphragm muscle fibers (*chapter 2*). Unexpectedly, this was not accompanied by a decrease in diaphragm fiber titin content. Under certain conditions, posttranslational modifications of the PEVK segment of titin may occur, which affect its passive elastic properties [35, 36]. Indeed, we found that the effects of mechanical ventilation on passive force could be imitated by dephosphorylation of titin through incubation of diaphragm muscle fibers with enzyme phosphatase-1 (*chapter 2*). As Hooijman et al. have demonstrated that active muscle force is decreased in diaphragm fibers of critical ill patients [4], it would be interesting to investigate if suboptimal sarcomeric stability plays a role in this process. Therefore, phosphorylation status of titin in diaphragm muscle biopsies of critically ill patients should be further investigated. If titin phosphorylation status is indeed increased in the critical ill and contributes to respiratory muscle dysfunction, it gives further directions to combat the development of ICU acquired respiratory muscle weakness.

Inflammatory pathways and respiratory muscle weakness

Inflammatory pathways have been linked to the development of respiratory muscle weakness [13, 37]. In this thesis, we show that IL-6 concentrations were elevated in plasma from septic patients (*chapter 3*). Moreover, it was significantly associated with the severity of contractile protein loss in skeletal myotubes. Importantly, adding IL-6 antibody dampened the loss of myosin in myotubes

exposed to plasma of septic patients. These data indicate a key role for IL-6 in inducing skeletal muscle atrophy during septic shock (*chapter 3*). It should be noted that when IL-6 was added to plasma of healthy subjects (in vitro), this did not induce a loss of contractile proteins in myotubes. Thus, although IL-6 has a prominent role in the atrophic response, other ligands appear required to induce atrophy. The important role for IL-6 in skeletal muscle atrophy is also supported by the fact that we found increased levels of IL-6 in diaphragm of mechanically ventilated rodents developing atrophy (*chapter 4 & 5*). Further supporting the role for IL-6 is that the attenuation of atrophy in TLR4 deficient ventilated animals was accompanied by a decrease of diaphragmatic IL-6 levels in these animals. These data (*chapter 3, 4 & 5*) are largely in line with previous studies, that found that IL-6 can induce an atrophic response in skeletal muscle [38-40]. It is important that other plasma factors are identified that play a role in the development of atrophy (*chapter 3).* Interestingly, LPS has the properties to activate TLR4 and we demonstrate in this thesis, that activating TLR4 induces an atrophic response in the diaphragm (*chapter 4).* It is plausible that in critically ill patients with septic shock, circulating LPS ligands concurrently with IL-6 induce an atrophic response in skeletal muscles. In conclusion, attenuating the inflammatory response by TLR4 deficiency or by hypercapnia might protect the diaphragm from atrophy during mechanical ventilation (*chapter 4 & 5*). The present results support the concept that cytokines provoked by mechanical ventilation are associated with diaphragm contractile protein loss. Therefore, further research in critically ill patients is needed to test the hypothesis that acting against specific points of inflammatory pathways, like IL-6 and TLR4, protects against respiratory muscle atrophy and weakness.

Mechanism and therapeutic applicability of interventions

Levosimendan

Respiratory muscle weakness is a major clinical problem in the critically ill patient. Yet, no adequate therapeutic intervention is currently available to improve respiratory muscle function (*reviewed in chapter 7*). Levosimendan has shown to affect several intracellular pathways involved in inflammation and oxidative stress [41, 42], besides its inotropic effects mainly through calcium sensitization [43, 44]. In this thesis, levosimendan administration in mechanically ventilated endotoxemic rodents reduced protein nitrosylation and markers of oxidative stress (*chapter 6*). Both oxidative stress and nitrosative stress have been linked in models of mechanical ventilation and experimental sepsis to a deterioration of diaphragm function [22, 45-47]. Therefore, levosimendan's proposed beneficial role through decreasing diaphragm nitrosative and oxidative stress during mechanical ventilation could protect diaphragm function under certain

conditions (i.e. presence of inflammation). Indeed, the data in the present thesis indicate that the beneficial effects of levosimendan are not only the result of calcium sensitization [43]. To speculate about further directions for research, levosimendan should be evaluated in the mechanically ventilated critically ill patients with sepsis to give more definitive answers if respiratory muscle function is indeed protected by decreased nitrosylation and oxidation of the respiratory muscles.

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Chapter 9

Samenvatting & Discussie

SAMENVATTING

Respiratoire spierzwakte komt regelmatig voor bij de kritisch zieke patiënt en is sterk geassocieerd met een verhoogde morbiditeit en mortaliteit. Eerder werd vastgesteld dat skeletspiervezel atrofie, verminderde eiwitsynthese en disfunctie van de resterende contractiele eiwitten ten grondslag ligt aan respiratoire spierzwakte bij kritisch zieke patiënten. Echter, de onderliggende pathofysiologie is grotendeels onbekend. De studies, beschreven in dit proefschrift, zijn ontworpen om ons begrip, over hoe respiratoire spierzwakte zich ontwikkelt in de kritisch zieke patiënt, te verbeteren. Ook is onderzocht of ademhalingsspierzwakte na mechanische ventilatie kan worden tegengegaan middels specifieke therapeutische strategieën.

De in ***hoofdstuk 2*** gepresenteerde studie laat zien dat 18 uur beademing van proefdieren de kracht genererende capaciteit van diafragmavezels vermindert. Verlies van kracht gaat gepaard met een vermindering van de concentratie van het skeletspiereiwit myosine in deze spiervezels. Naast actieve kracht, kan passieve kracht in spiervezels worden gemeten door het oprekken van de spiervezel. Passieve elastische eigenschappen van skeletspieren zijn onmisbaar voor de structurele stabiliteit en een optimale actieve krachtgeneratie. Passieve krachtgeneratie tijdens het oprekken van spiervezels was significant verlaagd in de vezels van beademde ratten. Echter, de concentratie van titine - het belangrijkste eiwit dat verantwoordelijk is voor passieve elastische eigenschappen van de skeletspier - was niet verschillend tussen de beademde groep en de controlegroep. Omdat we sterke aanwijzingen hadden dat de effecten van mechanische ventilatie op de reductie van passieve kracht kan worden nagebootst door defosforylatie van het elastische eiwit titine, hebben we de mate van defosforylering van titine bestudeerd. Defosforylering van titine middels fosfatase-1 verlaagde de passieve krachtgeneratie tijdens rek in diafragmavezels van controle, maar niet van geventileerde ratten. Dit geeft aan dat de mate van fosforylering van titine in diafragmavezels van mechanisch geventileerde dieren al laag was. We concluderen hieruit dat mechanische ventilatie de fosforylering status van titine vermindert in diafragma vezels, hetgeen resulteert in verminderde elasticiteit (“stijfheid”). Onze resultaten zijn van belang, aangezien verondersteld wordt dat normale elastische eigenschappen van spiervezels belangrijk zijn om de structurele integriteit van de spiervezels te waarborgen. Interessant is dat mechanische ventilatie geen invloed heeft op actieve of passieve kracht generatie van de soleus spier, wat aangeeft dat de ademhalingsspieren meer vatbaar zijn voor de schadelijke effecten van inactiviteit.

Verschillende studies suggereren dat activering van ontstekingsreacties (inflammatie) een rol speelt bij de ontwikkeling van spieratrofie en bijdragen aan respiratoire spierzwakte in de kritisch zieke patiënt. Inderdaad is eerder activering van inflammatie in de ademhalingsspieren aangetoond in diermodellen voor inflammatie. Het is echter onbekend of de inflammatoire reactie in de spier secundair is aan systemische inflammatie of het gevolg is van een lokale inflammatoire reactie. In ***hoofdstuk 3*** is daarom onderzocht of plasma, afkomstig van patiënten met een septische shock, een atrofische respons in gekweekte spiervezels (myotubes) kan induceren. Dit zou de hypothese dat systemische inflammatie bij deze patiënten bijdraagt aan spieratrofie ondersteunen. Myosine concentratie was ~ 25% lager in skelet myotubes welke blootgesteld waren aan het plasma van patiënten met een septische shock in vergelijking met myotubes die blootgesteld waren aan plasma van gezonde controles. In myotubes die blootgesteld waren aan septische shock plasma, was de concentratie geubiquitineerd myosine verhoogd. Deze stijging was parallel aan een in deze myotubes verhoogde expressie van de E3 ligasen MuRF-1 en MAFbx. In myotubes blootgesteld aan plasma van patiënten met een septische shock hebben we bewijs gevonden voor activatie van inflammatie, namelijk door activering van NF-κB, een belangrijke transcriptiefactor voor inflammatoire cytokines. Bovendien waren de verhoogde concentraties van IL-6 in het plasma van patiënten met een septische shock geassocieerd met de ernst van myosine verlies in de myotubes. Toevoegen van anti-IL6 aan plasma van patiënten met een septische shock voorkomt deels dat er verlies van myosine optreedt in deze myotubes. Deze resultaten geven aan dat plasma van patiënten met een septische shock, skeletspier atrofie kan induceren door activering van ontstekingsreacties met een centrale rol voor IL-6. Deze uitkomsten ondersteunen een rol voor systemische inflammatie in de ontwikkeling van respiratoire spieratrofie bij de kritisch zieke patiënt.

In ***hoofdstuk 4*** is de rol van toll like receptor4 (TLR4) en de ontwikkeling van atrofie als gevolg van gecontroleerde mechanische ventilatie onderzocht. Ook zijn inflammatoire reacties als gevolg van mechanische ventilatie bestudeerd. Hiertoe zijn wild-type muizen en TLR4 knock-out muizen onderworpen aan een gecontroleerde mechanische ventilatie gedurende 8 uur. In wild-type muizen resulteerde mechanische ventilatie in een significante afname van de myosine concentratie in het diafragma. Mechanische ventilatie had daarentegen geen invloed op de myosine concentratie in TLR4 knock-out muizen. De pro-inflammatoire cytokines IL-6 en keratinocyte-derived chemokine waren significant verhoogd in het diafragma van geventileerde wild-type muizen, maar niet in TLR4 knock-out muizen. Bovendien was light chain 3B-II, een marker voor autofagie, verhoogd na mechanische ventilatie in het diafragma van wild-type,

maar niet in TLR4 knock-out dieren. Deze studie toont aan dat TLR4 signalering een rol speelt bij de ontwikkeling van respiratoire spieratrofie, waarschijnlijk via activatie van inflammatoire cascades en van lysosomale autophagy.

Hypercapnische acidose is een geaccepteerde bijwerking van long protectieve ventilatie bij patiënten met het acute respiratory distress syndrome. Behalve een neveneffect van long beschermende ventilatie, wordt gesuggereerd dat hypercapnische acidose gunstige effecten kan hebben, bijvoorbeeld door het dempen van systemische en lokale (bijvoorbeeld long) inflammatoire reacties. Aangezien we aangetoond hebben dat activering van inflammatoire cascades waarschijnlijk een rol speelt bij respiratoire spieratrofie (hoofdstuk 3 en 4), hebben we besloten om in ***hoofdstuk 5*** te onderzoeken wat de effecten van hypercapnie op atrofie en de functie van de ademspieren zijn tijdens gecontroleerde mechanische ventilatie. Gezonde proefdieren zijn gecontroleerd beademd in de af- of aanwezigheid van hypercapnie. In lijn met eerdere studies vonden we dat de concentratie myosine werd verlaagd in geventileerde controle dieren, maar niet in de hypercapnische dieren. Hypercapnische acidose verminderde tevens de inflammatoire response die het gevolg is van beademing. De kracht producerende capaciteit was verlaagd in de diafragma spiervezels van beademde dieren, en onverwachts ook, zij in mindere mate, bij hypercapnische geventileerde dieren. Aangezien hypercapnie verlies van myosine voorkomt, maar geen beschermend effect heeft op de ontwikkeling van spierzwakte, hebben we de betrokkenheid van eiwitmodificaties door oxidatieve reacties onderzocht. Spiervezels werden geïncubeerd met de antioxidant dithiothreitol, waarna vervolgens contractiliteit werd bepaald. Dithiotreïtol herstelde de contractiliteit in diafragma spiervezels van hypercapnische geventileerde dieren, maar niet in normaal geventileerde dieren. Dit geeft aan dat oxidatieve eiwitmodificaties een rol spelen bij verlies van kracht ten gevolge van gecontroleerde mechanische ventilatie onder hypercapnische omstandigheden. Deze studie geeft aan dat de effecten van hypercapnie op de ademhalingsspieren complex zijn. Hoewel verlies van eiwit wordt voorkomen, ontstaat nog steeds zwakte.

Het cardiale inotrope middel levosimendan verbetert hartspier contractiliteit door optimalisering van de gevoeligheid van troponine voor calcium. Er is gesuggereerd dat levosimendan ook anti-inflammatoire en anti-oxidatieve eigenschappen heeft. Aangezien we een belangrijke rol voor inflammatoire cascades in de ontwikkeling van respiratoire spierzwakte en atrofie hebben gevonden tijdens mechanische ventilatie (hoofdstuk 3, 4 en 5), hebben we in ***hoofdstuk 6*** onderzocht of levosimendan inflammatie en oxidatie in het diafragma kan moduleren. Om een systemische inflammatoire reactie op te

wekken, zijn muizen blootgesteld aan lipopolysaccharide (LPS). Vervolgens zijn de dieren verdeeld in twee groepen: placebo en levosimendan. In het diafragma van muizen die met levosimendan waren behandeld vonden we een vermindering van genitrosyleerde eiwitten en ook een vermindering van markers voor oxidatieve stress. In tegenstelling tot eerdere studies door anderen, hebben we geen effect van levosimendan op pro-inflammatoire cytokines gevonden. Deze studie toont aan dat levosimendan kan beschermen tegen nitrosatieve stress en oxidatieve stress in de ademhalingsspieren van endotoxemische muizen, echter zonder modulatie van de inflammatoire reactie.

DISCUSSIE

Diafragma spieratrofie

Beademing is een levensreddende interventie bij patiënten met acute respiratoire insufficiëntie, maar kent ook bijwerkingen zoals longschade en disfunctie van de ademhalingsspieren. Het proefschrift richt zich op de effecten van beademing op de ademhalingsspieren. De pathofysiologie die ten grondslag ligt aan beademings-geïnduceerde ademhalingsspier disfunctie is heden onvolledig begrepen. Studies in dit proefschrift laten zien dat de dwarsdoorsnede (oppervlakte) van diafragma vezels is afgenomen na 18 uur beademing (*hoofdstuk 2*). Naast afname in de dwarsdoorsnede, vonden we na respectievelijk 18 en 8 uur beademing een verminderde concentratie myosine in het diafragma van ratten (*hoofdstuk 2*) en muizen (*hoofdstuk 4*). Deze resultaten zijn in lijn met eerdere bevindingen bij zowel mensen als proefdieren. Een belangrijke studie van collega Levine meldde dat beademing van hersendode patiënten al na 18-69 uur resulteert in atrofie van spiervezels uit het diafragma. Dit artikel suggereert dat diafragma atrofie zich snel ontwikkelt bij de mens wanneer deze spier geïnactiveerd wordt. Studies in dit proefschrift tonen aan dat de concentratie myosine in diafragma vezels van beademde dieren verlaagd is, hetgeen suggereert dat de ernst van verlies van contractiel eiwit groter is, dan de ernst van diafragma vezel atrofie (*hoofdstuk 2*). Uiteindelijk is spieratrofie het gevolg van een disbalans tussen proteolyse en eiwitsynthese. De ubiquitine-proteasoom cascade lijkt een belangrijke rol te spelen bij de verhoogde proteolyse van het diafragma tijdens beademing en ontsteking (inflammatie). Kort samengevat: dit traject wordt gekenmerkt door activering van twee belangrijke enzymen, de E3-ligases MuRF-1 en MAFbx. Deze enzymen markeren eiwitten voor degradatie door het proteasoom door middel van toevoegen van een ubiquitine-molecuul aan het te degraderen eiwit. Recente observaties in kritisch zieke beademde patiënten bevestigde een belangrijke rol van de ubiquitine-proteasoom cascade. In deze studie werd in diafragma biopten van deze patiënten een verhoogde activiteit van de ubiquitine-proteasoom cascade gevonden. Dit ging gepaard met tekenen van diafragma atrofie en verminderde kracht- generatie van diafragma vezels. In dit proefschrift wordt aangetoond dat circulerende liganden in het plasma van patiënten met een septische shock spieratrofie in gekweekte spiervezels (myotubes) kan induceren (*hoofdstuk 3*). Het verlies van contractiel eiwit gaat gepaard met een verhoogde expressie van de E3 ligases MuRF-1 en MAFbx en verhoogde concentratie van geubiquitineerd myosine. Activering van MuRF-1 kan worden gereguleerd door transcriptiefactor NFκB. Inderdaad induceert plasma van patiënten met een septische shock een verhoogde activiteit van NFκB in de myotubes (*hoofdstuk 3*). Conclusie: plasma van patiënten met een septische shock induceert spieratrofie in myotubes door

activering van de ubiquitine-proteasoom cascade alsook inflammatoire routes (*hoofdstuk 3*). Dit is een belangrijke bevinding omdat het voor het eerst laat zien dat liganden in plasma van patiënten met een septische shock atrofische cascades kan activeren in "gezonde" spiervezels. Op basis van deze data kan worden verondersteld dat circulerende factoren in het plasma van patiënten met septische shock atrofie induceert van perifere spieren en ademspieren. Verder onderzoek moet zich richten op identificatie van deze specifieke factoren en op strategieën die betreffende factoren inactiveren.

De rol van TLR4

Toll-like receptoren (TLR's) zijn essentieel in het afweersysteem vanwege de herkenning van specifieke liganden, zoals microbiële producten, maar ook van eiwitten die vrijkomen uit weefsel dat beschadigd is. De expressie van het subtype TLR4 is aangetoond in myotubes en diafragma weefsel. In dit proefschrift is de rol van TLR4 op de ontwikkeling van beademings-geïnduceerde diafragma atrofie bestudeerd (*hoofdstuk 4*). Bij muizen met TLR4 deficiëntie is de ernst van diafragma atrofie geïnduceerd door 8 uur gecontroleerde beademing minder ernstig dan in TLR-4 sufficiënte muizen. Eerder werd reeds aangetoond dat activatie van TLR4 in het diafragma door de specifieke ligand lipopolysaccharide (LPS), de expressie van pro-inflammatoire cytokines verhoogt en de diafragma functie vermindert. Activatie van de TLR4 receptor kan resulteren in activering van zowel de ubiquitine-proteasoom cascade als ook de lysosomale-autofagie cascade in skeletspieren. In dit proefschrift is geen verschil gevonden in activatie van de ubiquitine-proteasoom tussen beademde wild-type dieren en TLR4 deficiënte dieren. Dat geeft aan dat in dit model de ubiquitine-proteasoom cascade geen belangrijke rol speelt bij de ontwikkeling van atrofie. Dit is deels, in tegenstelling tot voorgaande studies die wel een associatie tussen beademing en het ontstaan van diafragma atrofie toonden, het gevolg van de activering van de ubiquitine-proteasoom cascade bij patiënten als ook bij dieren. Een rol van de ubiquitine-proteasoom cascade tijdens beademings-geïnduceerde diafragma atrofie - in het bijzonder na langere duur van beademing - kunnen we daarom niet uitsluiten. In dit proefschrift beschrijven we dat de autofagie marker LC3B-II is verhoogd in beademde wild-type dieren, maar niet in TLR4 deficiënte dieren (*hoofdstuk 4*). Derhalve suggereren deze bevindingen dat het verlies van contractiel eiwit via de TLR4 het gevolg is van activering van de lysosomale autofagie route. Deze data worden ondersteund door eerder werk van Doyle, waaruit blijkt dat de TLR4 agonist LPS, lysosomale autofagie in myotubes induceert. Ook werd in beademde patiënten activering van de lysosomale autofagie route, met daarop volgend diafragma atrofie, gevonden. Concluderend kunnen we stellen, dat beademing verlies van contractiele eiwitten kan induceren door activering van

TLR4. Het is interessant dat TLR4 liganden autofagie kan induceren in de spier. Dit proefschrift geeft dan ook een rationale om verder te onderzoeken of het blokkeren van de TLR4 route bij de beademde patiënt ademspierzwakte kan verminderen.

Diafragma functie en beademing

Het is algemeen geaccepteerd dat beademing het kracht genererende-vermogen verminderd van het diafragma in diermodellen en bij mensen. Vermindering van het kracht genererend vermogen kan theoretisch het resultaat zijn van ofwel verlies van contractiel eiwit of disfunctioneel contractiel eiwit. Hoewel atrofie is aangetoond in het diafragma van beademde dieren, is de rol van disfunctioneel contractiel eiwit grotendeels onbekend. Dit proefschrift beschrijft studies welke de rol van de kracht genererende capaciteit in gepermeabiliseerde diafragma vezels onderzoekt (*hoofdstuk 2*). Gepermeabiliseerde vezels zijn een uitstekend model om contractiel eiwit functie in vitro te bestuderen, aangezien de activering niet afhankelijk is van membraankenmerken of calciumhuishouding. Wij ontdekten dat beademing de kracht generatie verminderde in diafragma vezels in proportie tot de vermindering in vezeloppervlakte ("cross sectional area") (*hoofdstuk 2*). Dat geeft aan dat het resterend contractiel eiwit een normale kracht genererende capaciteit heeft. Dit is duidelijk in contrast met andere studies die melden dat er vermindering is van kracht genererende capaciteit van spiervezels, ook na correctie voor spiervezel oppervlakte. Bijvoorbeeld in een recente studie bij een heterogene groep van kritisch zieke patiënten bleek dat krachtafname van diafragma vezels niet alleen werd veroorzaakt door atrofie, maar dat deze ook het gevolg is van disfunctie van resterend contractiel eiwit. Hierbij moet wel worden opgemerkt dat bij kritisch zieke patiënten verschillende co-factoren een rol kunnen spelen naast beademing, waaronder toediening van bepaalde geneesmiddelen en infecties. Onlangs is aangetoond dat de "calcium sensitizer" levosimendan de ademspierfunctie in gezonde vrijwilligers verbetert. Bovendien is zeer recentelijk aangetoond dat de troponine activator CK-2066260 de kracht verbetert in spiervezels van beademde IC-patiënten. Concluderend kunnen we stellen, dat dit proefschrift duidelijk aantoont dat beademing verlies van contractiel eiwit in de ademhalingsspieren veroorzaakt, hetgeen resulteert in een vermindering van kracht-genererend vermogen van de spier. In tegenstelling tot studies bij patiënten hebben we geen bewijs voor disfunctionerend contractiel eiwit gevonden. Echter, in de kritisch zieke patiënt kunnen andere factoren, zoals bijvoorbeeld sepsis en geneesmiddelen, de functie van de resterende contractiele eiwitten aantasten. Daarom is verder onderzoek nodig naar mogelijke strategieën om ademspierzwakte tegen te gaan bij de IC patiënt. Deze interventies moeten niet alleen gericht zijn op vermindering van atrofische reacties, maar ook op

verbetering van contractiel eiwit functie. Recente en lopende onderzoeken met een calcium sensitizer of troponine activator zijn daarom van belang.

De rol van Hypercapnie

Bij ernstig zieke patiënten kan hypercapnische acidose ontstaan als gevolg van long protectieve beademing die gekenmerkt wordt door gebruik van lage teugvolumes. In dit proefschrift onderzochten we de effecten van hypercapnische acidose op de ademspieren (*hoofdstuk 5*). We vonden dat hypercapnie beschermende effecten heeft op het diafragma tijdens beademing, met name behoud van de myosine concentratie en daarmee remmen van de ontwikkeling van atrofie. Tot onze verrassing was er geen beschermend effect van hypercapnie op de contractiliteit van de ademspieren. Eerdere studies toonden aan dat beademing onder hypercapnische omstandigheden beschermt tegen het ontwikkelen van ademspierzwakte. De resultaten van het onderzoek, zoals beschreven in dit proefschrift, tonen aan dat bij hypercapnisch beademde dieren contractiel eiwit disfunctioneel is als gevolg van posttranslationele modificatie van betreffende eiwitten. Inderdaad kan het antioxidant dithiotreitol de diafragma functie herstellen in vezels van hypercapnisch beademde dieren. Dit geeft aan dat oxidatieve modificatie van contractiel eiwit een belangrijke rol speelt tijdens beademing onder hypercapnische omstandigheden. Inderdaad is eerder aangetoond dat CO_2 vorming van oxidanten kan verhogen in het diafragma. Onze resultaten tonen aan dat verdere studies zich moeten richten op het gecombineerde effect van hypercapnie en antioxidanten op spierfunctie tijdens beademing.

Passieve kracht van het diafragma

Passieve elastische eigenschappen zijn essentieel voor structurele stabiliteit en optimale actieve functie van spiervezels. Titine is het belangrijkste eiwit dat passieve elastische eigenschappen in skeletspiervezels bepaalt. Beademing vermindert de passieve kracht eigenschappen van diafragma vezels (*hoofdstuk 2*). Dit ging overigens niet gepaard met een afname van de titine concentratie in diafragma vezels. Onder bepaalde omstandigheden kunnen post-translationele modificaties ontstaan van het PEVK segment van het titine molecuul, die invloed hebben op de passief elastische eigenschappen. Inderdaad vonden we dat de effecten van beademing op passieve kracht kon worden nagebootst door defosforylering van titine. In de studie in dit proefschrift werd defosforylering geïnduceerd door incubatie van diafragma vezels met het enzym fosfatase-1 (*hoofdstuk 2*). Hooijman heeft eerder aangetoond dat actieve spierkracht is verminderd in diafragma vezels van kritische zieke patiënten. Het zou interessant zijn om te onderzoeken of suboptimale spiervezel stabiliteit (door veranderingen in eigenschappen van titine) een rol speelt in dit proces. Daarvoor moet de fosforylering status van

titine in diafragma van spierbiopten van ernstig zieke patiënten nader worden onderzocht. Indien titine's fosforyleringsstatus inderdaad verhoogd is bij de kritisch zieke patiënt en daarmee bijdraagt aan ademspierdisfunctie, zou dit verdere richting geven hoe ademspierzwakte te verminderen of te voorkomen is bij onze patiënten.

Inflammatoire routes en ademhalingsspierzwakte

Eerdere studies toonden aan dat inflammatoire reacties een rol kunnen spelen bij de ontwikkeling van ademspierzwakte. In dit proefschrift laten we zien dat de concentratie van IL-6 verhoogd is in het plasma van patiënten met een septische shock (*hoofdstuk 3*). Bovendien was dit significant geassocieerd met de ernst van verlies van contractiel eiwit in myotubes. Belangrijk was dat de toevoeging van een IL-6 antilichaam verlies van myosine vermindert in myotubes welke blootgesteld waren aan plasma van septische patiënten. Deze resultaten wijzen op een belangrijke rol voor IL-6 bij het induceren van skeletspier atrofie tijdens een septische shock (*hoofdstuk 3*). Wanneer echter IL-6 werd toegevoegd aan plasma van gezonde personen, gaf dit geen verlies van contractiel eiwit in myotubes. Ofschoon IL-6 een belangrijke rol speelt bij de atrofische reacties, zijn andere moleculen nodig voor het ontstaan van atrofie. De belangrijke rol van IL-6 in skeletspieratrofie wordt ondersteund door het feit dat we verhoogde concentraties van IL-6 hebben gevonden in het diafragma van beademde dieren, die atrofie ontwikkelen (*hoofdstuk 4 en 5*). Verdere ondersteuning voor de rol van IL-6 is dat de verminderde atrofische response in beademde TLR4 deficiënte dieren gepaard gaat met een afname van de concentratie van IL-6 in het diafragma van deze dieren. Deze data (*hoofdstuk 3, 4 en 5*) worden ondersteund door eerdere studies waaruit bleek dat IL-6 een atrofische reactie in skeletspieren kan opwekken. Het is belangrijk dat andere factoren in plasma geïdentificeerd worden die een rol spelen bij de ontwikkeling van atrofie (*hoofdstuk 3*). Zoals eerder genoemd, kan LPS de TLR4 activeren. In dit proefschrift laten we zien dat het activeren van TLR4 leidt tot een atrofische respons in het diafragma (*hoofdstuk 4*). Het is aannemelijk dat bij ernstig zieke patiënten met een septische shock, circulerende LPS liganden gelijktijdig met IL-6 een atrofisch reactie opwekt in skeletspieren. Concluderend kan gesteld worden dat, door het verminderen van een inflammatoire reactie door TLR4 deficiëntie of hypercapnie, dit bescherming kan bieden tegen diafragma atrofie geïnduceerd door beademing (*hoofdstuk 4 en 5*). Deze resultaten ondersteunen het concept dat aanwezigheid van cytokines in het diafragma tijdens beademing, geassocieerd worden met verlies van contractiel eiwit in het diafragma. Daarom is verder onderzoek nodig om het effect van modulatie van specifieke stappen in inflammatoire cascades, zoals IL-6 en TLR4, te onderzoeken op de ontwikkeling van atrofie en zwakte in ademspieren van beademde IC patiënten.

Mechanismen en therapeutische toepasbaarheid van interventies

Levosimendan

Ademspierzwakte is een klinisch relevant probleem voor de kritisch zieke patiënt. Toch is er geen adequate therapeutische interventie momenteel beschikbaar om de ademspierfunctie te verbeteren (*beschreven in hoofdstuk 7*). Eerdere studies in inflammatoire cellen suggereren dat levosimendan naast de bekende inotrope effecten ook inflammatie en oxidatieve stress kan moduleren. In dit proefschrift laten we zien dat toediening van levosimendan in beademde endotoxemische dieren leidt tot vermindering van genitrosyleerde eiwitten en markers van oxidatieve stress in het diafragma (*hoofdstuk 6*). Het is bekend dat zowel oxidatieve stress als nitrosatieve stress mogelijk een rol speelt bij de afname in diafragma functie tijdens beademing en/of experimentele sepsis. Vanwege de effecten van levosimendan op oxidatieve en nitrosatieve stress zou dit middel een beschermend effect kunnen hebben op het diafragma onder bepaalde condities (d.w.z. de aanwezigheid van inflammatie). De resultaten in dit proefschrift suggereren inderdaad dat levosimendan nitrosatieve stress tijdens beademing vermindert. Daarmee tonen wij aan dat de positieve effecten van levosimendan op ademspierfunctie niet alleen gemedieerd zijn via bekende calcium sensitizing, maar ook door modulatie van andere moleculaire pathways. In de toekomst moet het effect van levosimendan worden onderzocht op diafragmafunctie bij de beademde ernstig zieke patiënt met sepsis.

Dankwoord

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CURRICULUM VITAE

Willem-Jan, Wilhelmus Johannes Maria Schellekens, werd geboren op 11 mei 1984 te 's-Hertogenbosch. In 2002 behaalde hij zijn atheneum diploma aan het Maurick College te Vught. Van 2002 tot 2003 studeerde hij biologie en van 2003 tot 2004 biomedische wetenschappen aan de Radboud Universiteit Nijmegen. Beide studies sloot hij af met het propedeutisch examen. In 2004 mocht hij aanvangen met de studie geneeskunde aan de Radboud Universiteit Nijmegen. In het kader van zijn studie voerde hij in 2007 zijn onderzoeksstage uit op de afdelingen Anesthesiologie en Intensive Care (prof. G.J. Scheffer en dr. L.M.A. Heunks). Tijdens deze stage werden de eerste fundamenten van dit proefschrift gelegd in de vorm van pilot-experimenten met langdurig beademde proefdieren met als doel uiteindelijk de ademhalingsspieren te bestuderen. In 2009 behaalde hij, na het lopen van zijn co-schappen, zijn arts-examen (Radboud Universiteit Nijmegen). In 2010 is hij vervolgens te Nijmegen als arts-onderzoeker begonnen om aan zijn promotieonderzoek te werken, welke hij verder voorzette tijdens zijn opleiding tot anesthesioloog (hoofd: prof. G.J. Scheffer) en uiteindelijk resulteerde in dit proefschrift. Per 2015 rondde hij zijn opleiding tot anesthesioloog af en begon hij later dat jaar aan de opleiding tot intensivist (hoofd: prof. dr. J.G. v.d. Hoeven).
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